KINETICS OF ACETYLCHOLINESTERASE INHIBITION BY ESERINE AND A MONO-QUATERNARY PROPARGYLIC ETHER

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ABSTRACT

The kinetics of acetylcholinesterase-catalyzed hydrolysis of acetylthiocholine was studied polarographically. The Km and Vmax values were calculated at three different concentrations of ACHE and it was found that the system behaves according to the Michaelis - Menten formulation.

The inhibition of ACHE by eserine was studied at zero enzyme-eserine incubation time by using the polarographic and the spectrophotometric methods. The inhibition was found to be competitive, implying that no carbamylation of the enzyme takes place under these conditions. The two methods gave similar results excepting the percent inhibition and the K_i values. These differences were attributed to the differences in the ionic strengths used in these methods.

Iodide was found to interfere with the determination of thiocholine. A considerable inhibition of ATCH hydrolysis was observed in the presence of potassium iodide.

The inhibition of ACHE by 1-(4-dimethylamino-2-butynyloxy)-3-(2-propynyloxy)-bengene methiodide was studied by using the

polarographic method. After making corrections for iodide interference, the inhibition was found to be non-competitive. This is probably due to the interaction of the triple bond with the acidic group of the acetylated enzyme. Since the iodide interferes in the polarographic method, the action of this compound should be re-investigated by using the chloride salt.

ACKNOWLEDGEMENTS

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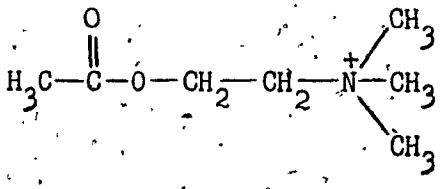
INTRODUCTION

Acetylcholinesterase (E.C.3.1.17) (1) is found in the nervous tissues of all species of animals (2). It has also been found in places other than conducting tissues where its physiological function and identity with neural acetylcholinesterase (ACHE) are uncertain. In man and most other mammals such ACHE is localized almost entirely in the erythrocytes, whereas the serum is rich in Cholinesterase (E.C.3.1.1.8).

The function of ACHE is to hydrolyze Acetylcholine (I) which is found in all types of nerves. Besides its hydrolytic activity there has been some suggestion that it may function as a physiological receptor (3).

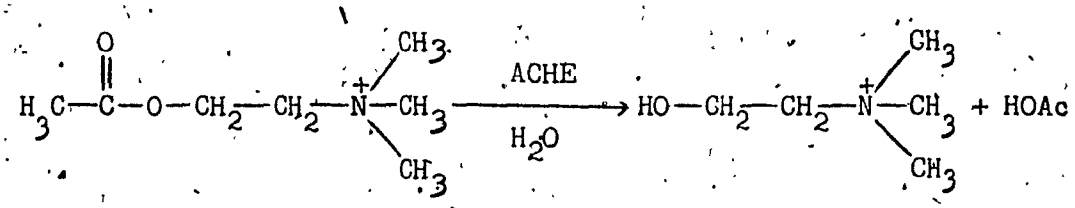


(I)

Acetylcholine (ACH) plays a leading role in the conduction of impulses by producing an electric potential which travels along the nerve cells during conduction. ACH produces this potential - via specific receptors as yet unknown - by causing a very fast change in the ion - permeability of the membrane surrounding the nerve cell (4). Due to this function ACH has been

variously named as action substance, transmitter substance, neuro - humoral substance, etc.

After its action the removal of ACH is very essential because before a second impulse can be transmitted the original state of the membranes must be re-established. The removal of ACH is achieved by ACHE which hydrolyzes it into choline and acetic acid. The hydrolytic reaction is given by scheme 1 below.



ACH Choline Acetic Acid

Scheme 1.

From what has been said it is clear that both the release and the removal of ACH is essential for impulse conduction. When the action of ACHE is blocked ACH accumulates, and the original state of the membrane cannot be re-established. This leads to severe and often fatal endogenous poisoning involving irritation and paralysis. Death is generally caused by respiratory paralysis (5).

Because of its importance in nerve activity a large number of compounds have been studied for their inhibitory

effects on ACHE. Most of these compounds are highly toxic and some anticholinesterases are used as war gases and as insecticides (6).

There are three main classes of anti - cholinesterases: 1) Organophosphates, 2) Carbamates, and 3) compounds containing positively charged nitrogen atom similar to the substrate, ACH.

Organophosphates and carbamates inhibit ACHE by phosphorylating and carbamylating, respectively, groups on the active site. The action of these compounds has been reviewed recently (6). The quaternary ammonium compounds show inhibition, in most cases, purely due to competition with the substrate for the active site of the enzyme. The inhibitory power of these compounds is reduced when the cationic group is converted into a neutral one (7).

Before discussing the mechanism of ACHE inhibition, a brief discussion of enzyme inhibition in general, of active centers, and about methods for measuring ACHE activity should be presented.

Enzyme Inhibition.

An inhibitor reduces the rate of enzyme catalysis whatever criterion is applied, whether it be utilization of substrate or formation of products. According to the conventional ideas of the action of enzyme inhibitors (8), the inhibition is classified as Competitive, Non - Competitive, or Mixed.

Pure competitive inhibition results when an inhibitor combines with the same enzyme site as does the substrate, thereby preventing the formation of a complex between enzyme and the substrate. These situations are shown in Scheme 2 and 3.

$$E + S \rightleftharpoons ES \qquad \text{Scheme 2}$$

$$E + I \rightleftharpoons EI \qquad \text{Scheme 3}$$

Where: E = free enzyme, S = substrate

ES = enzyme-substrate complex

EI = enzyme-inhibitor complex

Non - competitive inhibition results when the inhibitor and substrate may simultaneously combine with the enzyme, forming a ternary complex involving enzyme, substrate, and inhibitor as shown in Scheme 4 and 5. Only enzyme-substrate complex

$$ES + I \rightleftharpoons ESI \qquad \text{Scheme 4}$$

$$EI + S \rightleftharpoons EIS \qquad \text{Scheme 5}$$

which is free of combined inhibitor being able to form products. If the equilibrium constants for the combination of inhibitor with the free enzyme and enzyme-substrate complex are equal "simple" or "pure" non-competitive inhibition is observed. If, on the other hand, these equilibrium constants are different, a more complicated inhibition law is observed and the inhibition is sometimes described as "mixed".

The competitive and non-competitive inhibitors are recognized from the effect they have on the constants Km and Vmax of the Michaelis - Menten equation (9):

$$v = Vmax.S/Km + S \qquad (1)$$

This equation for the rate of an enzymatic reaction is based on the following scheme:

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_2} E + P$$

Scheme 6.

where:

v = observed rate $= k_2[ES]$

ES = Michaelis complex

E = free enzyme

Vmax = maximum theoretical rate $= k_2[E_o]$

E_o = total enzyme

k_1 = rate constant for the formation of ES

k_{-1} = rate constant for the breakdown of ES into free enzyme, E, and substrate, S

k_2 = rate constant for the breakdown of ES into free enzyme and products, P

P = products

$Km = k_1 + k_2/k_1$ (Michaelis constant)

S = Substrate

A competitive inhibitor increases Km value without affecting Vmax, whereas a non-competitive inhibitor decreases Vmax without affecting the Km value. A "mixed" inhibitor, on the other hand, affects both the Km and Vmax values.

The Km and Vmax values can be evaluated from the Lineweaver - Burke plot (10) which is based on equation 2, a linear transformation of Michaelis - Menten equation.

$$1/v = 1/Vmax + Km/Vmax \cdot 1/S \qquad (2)$$

There are other linear transformations of Michaelis - Menten equation and their statistical merits have been reviewed recently (11).

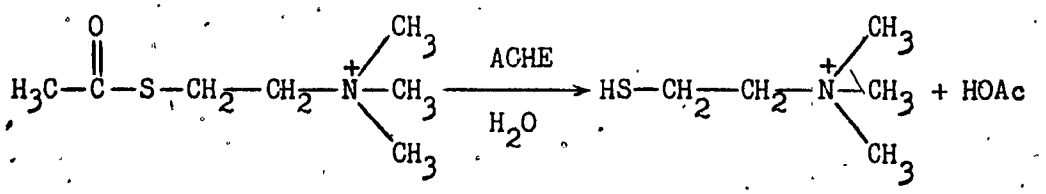
Methods for determining ACHE activity.

There are variety of methods available for measuring ACHE activity and these are described below briefly.

Hestrin's Method.(13). This method detects the amount of unchanged ACH based on the reaction of ACH with an alkaline solution of hydroxylamine to give acetylhydroxamic acid, whose ferric iron complex is then determined photometrically. This method is said to give values 15 - 20% low for the initial rate of the enzymatic reaction (14) and is therefore not suitable for kinetic investigations.

pH stat Method (15). In this method pH is kept constant by continuously neutralizing the acetic acid produced during the hydrolysis with NaOH solution, the titer being directly proportional to the amount of acetylcholine that is hydrolyzed by the enzyme.

Ellman's Method. (16). This method utilizes Acetylthiocholine (ATCH), an artificial substrate, which in a similar fashion to ACH is hydrolyzed to thiocholine and acetic acid. The hydrolysis of ATCH is shown below in scheme 7.



ATCH Thiocholine Acetic Acid

Scheme 7.

In this method, the production of thiocholine (TCH) is estimated by disulfide exchange with 5,5' - dithiobis - (2 - nitrobenzoic acid) which yields colored 5-thio-2-nitrobenzoate anion with a maxima at 412 nm.

Polarographic Method. (17). This method also utilizes acetyl - thiocholine and measures the production of thiocholine (TCH) which gives an anodic polarographic wave, typical of compounds containing SH-group. The reaction involved is:

$$(TCH)SH + Hg \rightleftarrows TCH(SHg) + H^+ + 1e$$

This method has been used in this project and will be discussed in detail later on.

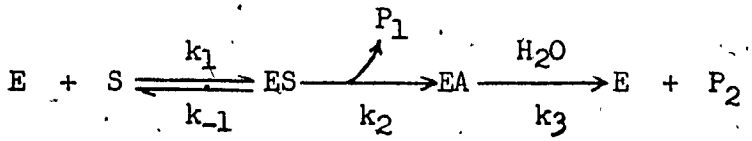
Further methods are based on the colorimetric determination of the slight pH change, which is caused by the production of acetic acid, by using indicators e.g. phenol red, cresol red, bromocresol purple, etc. (18).

Active Centers Of Acetylcholinesterase.

The active site of ACHE can be divided into two subsites, one, the esteratic site which binds the acetyl portion of ACH and about 5 Å away lies the second site, the anionic site. The quaternary nitrogen atom of choline as well as cationic inhibitors are attached to this anionic site. The other forces participating in the fixation of the substrate besides the coulombic ones are Van der Waals forces involving the methyl groups of choline.

The group or groups at the anionic site are unspecified at present but the esteratic site has been investigated in greater detail. At this site there are three active groups: a basic group, probably an imidazole nitrogen, at a distance of 5 Å from the anionic site, an acid group at a distance of about 2.5 Å from the anionic site and a serine hydroxyl group. Reviews of the active site of ACHE have appeared recently (19,20,21).

ACHE-catalyzed hydrolysis of substrate proceeds in three steps as illustrated in scheme 8.



Scheme 8.

Where: E = enzyme, S = ACH, ES = Michaelis complex, EA = acetylated enzyme, P_1 = choline, P_2 = acetic acid.

In the formation of Michaelis complex (ES scheme 8) both the sites, anionic and esteratic, are occupied but upon the loss of choline (P_1) the anionic site becomes free in the acetylated enzyme (EA). In EA, the acetate is attached to the hydroxyl group of serine. The next step, the deacetylation (k_3 in scheme 8), removes acetate and leaves a free enzyme.

In the case of ACH and ACTH, deacetylation is the rate determining step (22). The steady-state rate equation for the formulation of scheme 8 is given below. (23).

$$1/v = 1/kE_o + Km/kE_o \cdot 1/S \qquad (3)$$

Where: $Km = k_{-1} + k_2/k_1(1 + k_2/k_3)$

$Vmax = k[E_o]$

$k = k_2k_3/k_2 + k_3$

Mechanism Of ACHE Inhibition.

Only the action of reversible inhibitors will be discussed here. Irreversible inhibitors have been discussed recently.(24). Reversible inhibitors are those which form enzyme - inhibitor complexes that can dissociate (i.e. inhibitors can be separated from the enzyme e.g. by dialysis).

This group of inhibitors includes all substances that show a more or less strong affinity for the active

center of the enzyme, including therefore substances such as substrates, reactivators, and cleavage products. These substances are substituted derivatives of ammonium ion, which because of their resemblance to the substrate, become attached to the anionic site. Some compounds with a tertiary nitrogen atom also show inhibition.

Apart from adding to the anionic site of the free enzyme, the hydrolytic process (scheme 8) suggests that an inhibitor might also combine with the acetylated enzyme (EA in scheme 8), since in this the anionic site is free. If this is the case, the kinetics of inhibition would be expected to show competitive and non-competitive components. Indeed, it has been found that certain compounds (e.g. Cis-2-dimethyl-amino-cyclohexanol, Trimethylammonium ion, etc.) show non-competitive inhibition, whereas Eserine, Neostigmine, Carbachol, etc. show competitive inhibition. (22,25).

The difference in the modes of inhibition by these compounds was explained on the basis that the compounds inhibiting non-competitively do so by blocking deacetylation; the competitively inhibiting compounds, though attached to the acetylated enzyme, do not block deacetylation. (22,25). The requirement for blocking deacetylation was found to be the presence of a high electron density center at a distance of about 2.5 A from the cationic head. The blocking of deacetylation occurs due to the interaction of this high electron density center with the acidic group on the active site (25).

The reaction sequence of ACHE-catalyzed hydrolysis of the substrate in the presence of a reversible inhibitor is given below in scheme 9.

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow[k_2]{P_1} EA \xrightarrow[k_3]{} E + P_2$$

$$E + I \underset{k_{-i}}{\overset{k_i}{\rightleftharpoons}} EI \qquad K_i = k_{-i}/k_i$$

$$EA + I \underset{k'_{-i}}{\overset{k'_i}{\rightleftharpoons}} EAI \qquad K'_i = k'_{-i}/k'_i$$

Scheme 9.

The steady-state rate equation based on the above scheme is given in equation 4 below.

$$1/v = 1/kE_o\left[1 + [I]/K'_i(1 + k_3/k_2)\right] + Km/kE_o\left[1 + [I]/K_i\right] \cdot 1/S \qquad (4)$$

Where: $E_o = E + ES + EA + EI + EIA$

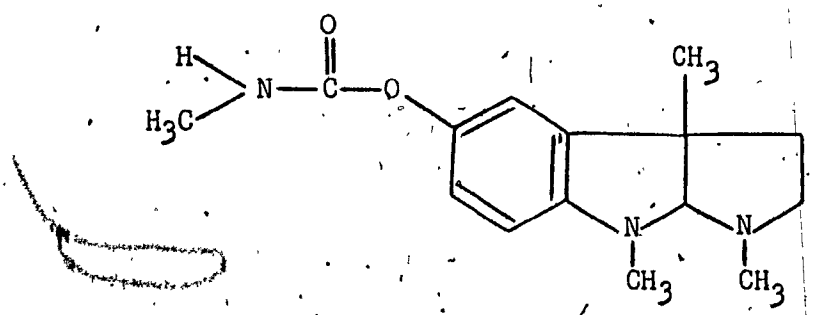
A non-competitive component is indicated by a displacement of the 1/v intercept. The size of the displacement depends on the value of k_3/k_2 and, therefore, on the substrate. If

k_3/k_2 were large (i.e. acetylation being the rate controlling step), a quite sizable inhibition of the deacetylation would not significantly decrease the over-all velocity.

This dependence on substrate has been observed (25). It was found that cis-2-dimethyl-amino-cyclohexanol inhibits the hydrolysis of methylamino acetate, an artificial substrate, for which $k_2 < k_3$ competitively. With ACH as the substrate, for which $k_2 > k_3$, this substance inhibits non-competitively. This shows that non-competitive inhibition results when an inhibitor interferes with the deacetylation step.

Inhibition by Eserine - Literature Survey.

Eserine (II) belongs to the group of compounds known as carbamates. Several compounds of this class e.g. Eserine, Neostigmine are very potent inhibitors of ACHE.



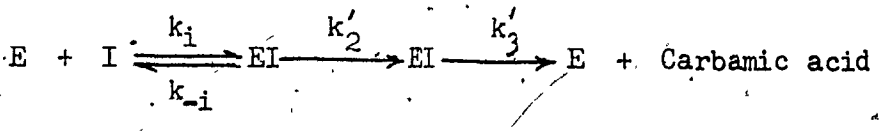
(II)

Inhibition by eserine has been studied for a long time by many workers and it was believed, up to recently, that it is a pure competitive inhibitor of ACHE(25,26). However, it has been noted that greater inhibition is obtained if the inhibitor is added to the enzyme before the substrate than if inhibitor and the substrate are added at the same time.

It was shown by Wilson et.al.(27) that eserine and other carbamates are not simple competitive inhibitors of ACHE but rather act as poor substrates and form carbamylated enzyme, like the acetylated enzyme. That the carbamates act by carbamylating the enzyme has been reported by other workers also (28,29,30).

The reaction sequence of carbamate action,

according to Wilson et.al. (27), is given in scheme 10 below.



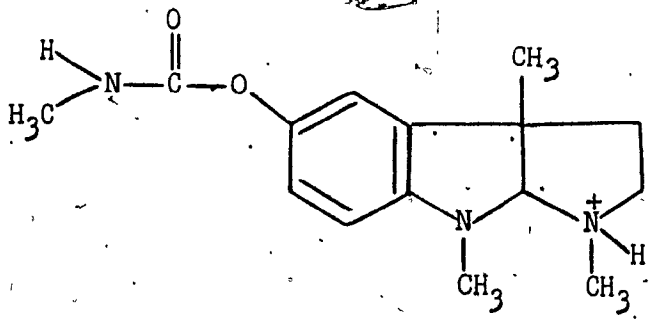
Where: I = Carbamate inhibitor
EI = enzyme-inhibitor complex
EI = carbamylated enzyme

Scheme 10.

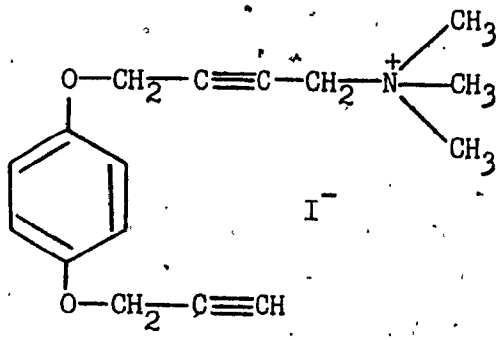
This reaction scheme is similar to substrate hydrolysis (scheme 8). However, the decarbamylation step (k'_3) is much slower than deacetylation.

The above scheme of the carbamate action implies that the inhibition by eserine should be non-competitive. It has been reported recently that if the enzyme and eserine are allowed to incubate for as little as 3 minutes, the inhibition is non-competitive. (31,32).

From the pH studies on the inhibition of ACHE by eserine it was concluded that the protonated form of eserine (III) is the enzyme inhibitor (33).



(III)



(IV)

In this project the inhibition of ACHE by eserine (II) and by 1 - (4 - dimethylamino - 2 - Butynyloxy) - 3 - (2 - Propynyloxy) - Benzene Methiodide (IV), a mono-quaternary propargylic ether. Both compounds were studied under zero pre-incubation time with the enzyme.

Eserine was chosen partly because of its controversial mode of inhibition and partly to establish whether or not the polarographic method can be used to study the inhibition of ACHE by highly potent inhibitors.

The acetylenic quaternary nitrogen compound (IV) was a newly synthesized compound. It was desired to study whether or not this compound has any anticholinesterase activity and, if it has, then what is the mode of inhibition.

In addition to inhibition studies, the effect of substrate concentration was also studied at different enzyme concentrations and the constants characterizing the hydrolytic reaction were evaluated.

METHODS AND MATERIALS

The rates of ACHE catalyzed hydrolysis of Acetylthiocholine were followed polarographically, measuring the production of Thiocholine (TCH), and spectrophotometrically, measuring the production of acetic acid by using phenol red as the indicator. These methods are discussed below.

Polarographic Method. This method is based on the fact that compounds containing -SH group yield an anodic polarographic wave. Thiocholine is such a compound and gives an anodic wave, typical of -SH group compounds, the diffusion current at the plateau being proportional to the concentration of TCH.

The diffusion current is given by the Ilkovic equation (34) as:

$$\bar{id} = 607\ n\ m^{2/3}\ \gamma^{1/6}\ D^{1/2}\ C \qquad (5)$$

where: $\bar{id}$ = the average diffusion current (in micro amperes, ua)

n = the number of electrons involved in the electrode reaction.

m = the mercury flow rate (mg/sec)

γ = the drop life (sec)

D = the diffusion coefficient (cm^2 /sec)

C = the concentration of the electroactive substance, TCH in this case, (mM)

By maintaining the drop life, the mercury

flow rate and the temperature constant, the Ilkovic equation reduces to:

$$id = K.C$$

i.e. the average diffusion current is a linear function of the concentration of electroactive substance

Fig.1 shows the polarographic waves of the ACHE-ATCH system. Curve No. 1 is the background current obtained with buffer solution and ATCH, Curves No. 2 - 5 were taken at an interval of 5 - 10 min after adding ACHE and show the anodic waves due to TCH.

Since the current at the plateau of the polarographic wave is proportional to thiocholine concentration, the production of TCH as a function of time can be followed by applying any potential within this plateau. In this study the applied potential used was -0.05 V vs Ag/AgCl, a value which falls on the plateau. The initial rates were calculated from the diffusion current - time recordings.

Fig.2 shows a typical current - time curve obtained by this method during the ATCH hydrolysis. The initial rates were measured from the initial linear portions of such curves (35) in ua/min. In the case of experiments at low substrate concentrations or with inhibitors, when the current - time curves did not show any linear portions, the initial rates were measured by drawing tangents to the curves at time t = 1 minute.

Fig. 1. Polarographic waves due to thiocholine - produced during the hydrolysis of Acetylthiocholine by Acetylcholinesterase.

Curve No. 1. Background current obtained with buffer and ATCH.

Curves No. 2 - 5. Same as curve No.1, except that ACHE added; each polarogram run at an interval of 5-10 min from the preceeding one.

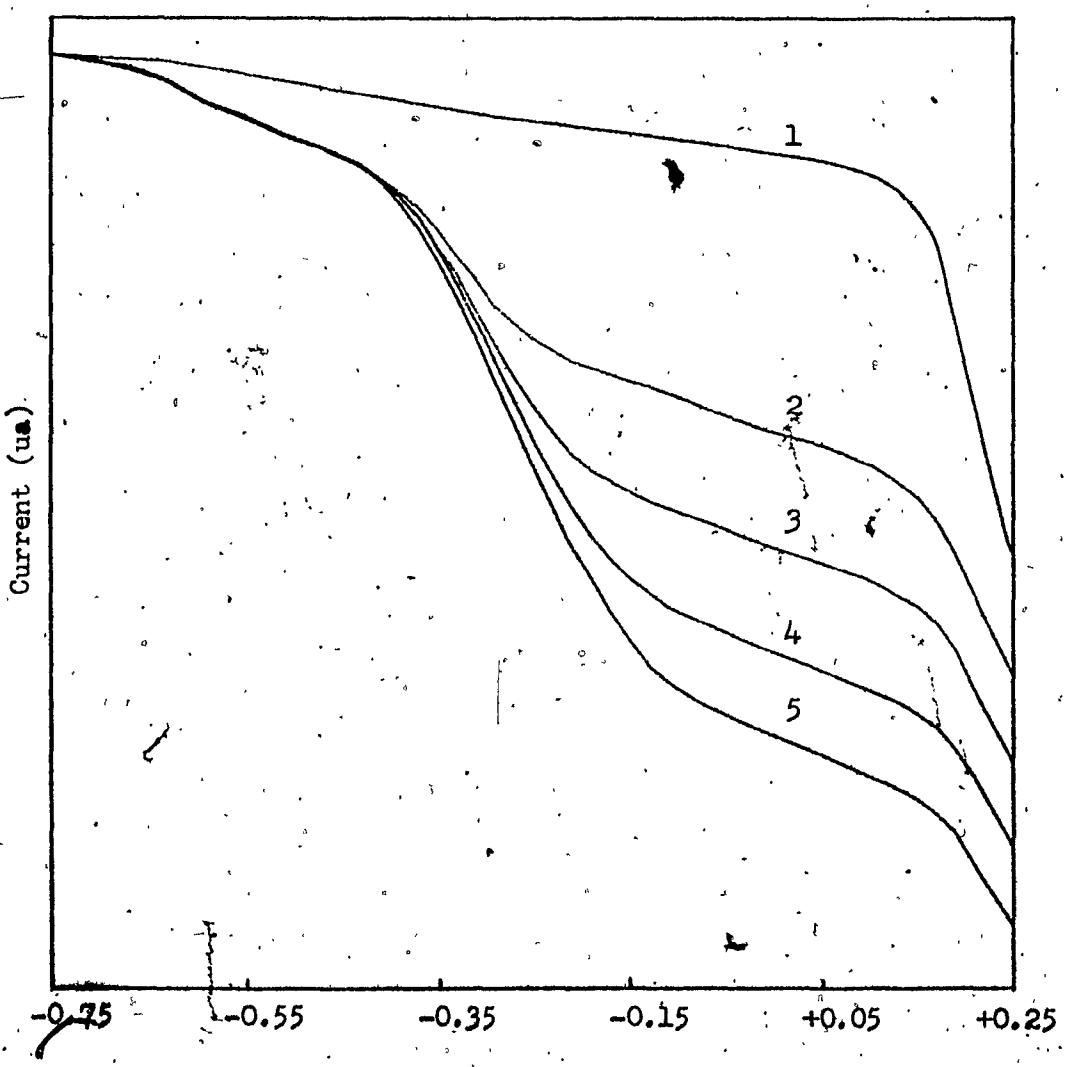
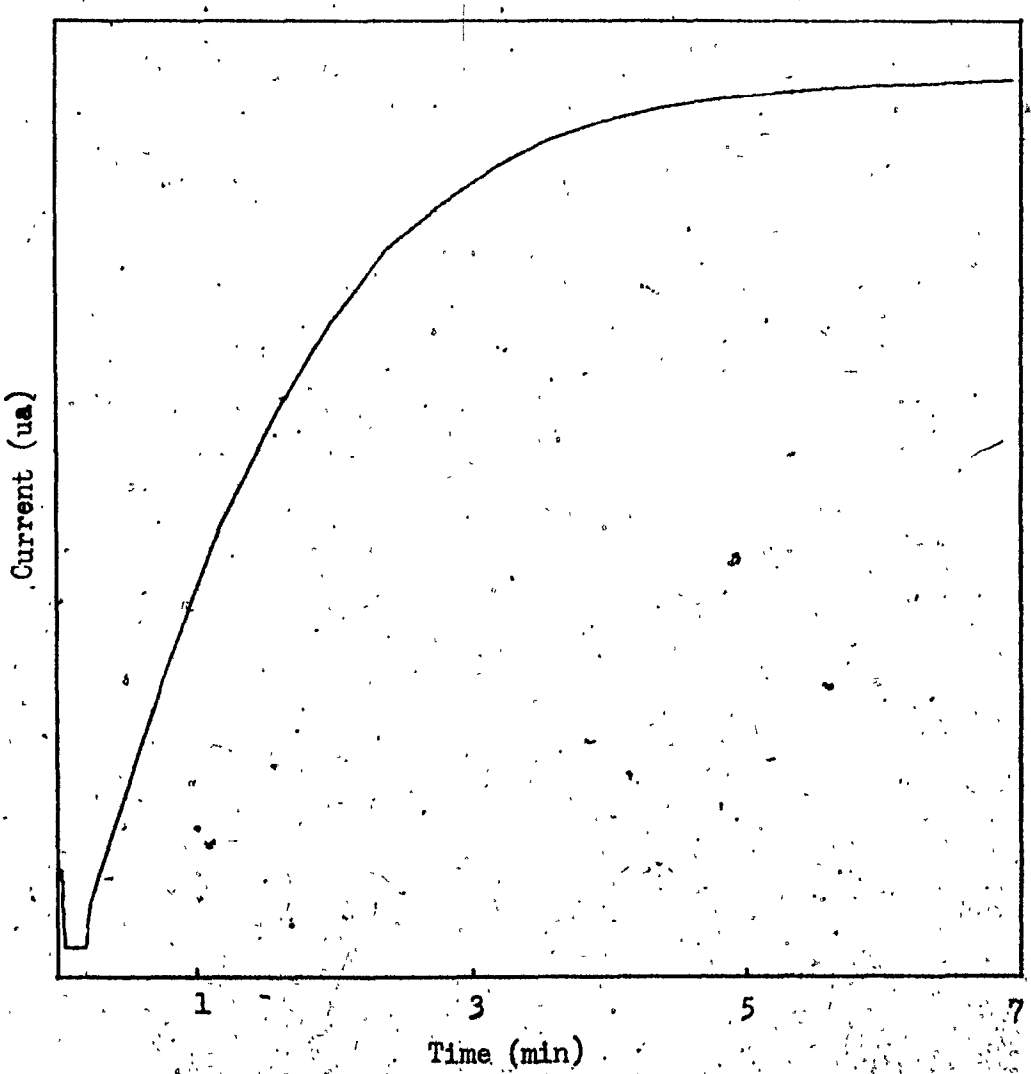


Fig. 2. Progress Curve Of Acetylthiocholine Hydrolysis By Acetylcholinesterase - Current-time curve.



Calibration Of The Polarograph.

The rate of thiocholine production measured in ua/min may be converted to umoles TCH/min by calibrating the polarograph with reduced Glutathione (GSH), which gives an anodic wave similar to thiocholine.(17). This involves the measurement of diffusion currents due to GSH standards at the potential chosen for measuring TCH production i.e. -0.05 V versus Ag/AgCl.

However, it was realized that calibration can also be made by Acetylthiocholine itself. This is based on the fact that the plateau of the current - time curve (Fig.2) represents the complete hydrolysis of ATCH to TCH and measurement of diffusion current at this point will be proportional to initial concentration of ATCH or concentration of TCH since 1 mole of ATCH is hydrolyzed to give 1 mole of TCH. (scheme 7). This procedure is based on the assumption that the hydrolysis of ATCH is complete and this can be ensured in the shortest possible time by taking an excess of enzyme.

Both Glutathione and Acetylthiocholine were used in calibration experiments and the factors obtained in each case are given below.

With GSH	$0.149 \pm 0.006^{*}$ ua/ 10^{-4} M GSH
With ATCH	$0.203 \pm 0.009^{*}$ ua/ 10^{-4} M TCH

* Standard deviation.

The experimental data are listed in appendix "B".

The factor obtained with ATCH has been used, for converting initial rates in ua/min into umoles TCH/min, instead of factor obtained with GSH due to the following reasons:

1. The value obtained with GSH differs considerably from the value of 0.20 ± 0.02 ua/10^{-4} M GSH obtained previously in this laboratory (45). The difference is probably due to the fact that GSH gets easily oxidized (46), therefore does not give very reproducible results.

2. The factor, 0.203 ± 0.009 ua/10^{-4} M TCH, obtained by using ATCH agrees very well with the value of 0.20 ± 0.02 ua/10^{-4} M GSH. Since the value with ATCH and the previous value with GSH are about the same, it seems that the GSH used in this study was not completely reduced.

3. The factor obtained with ATCH also agrees closely with the theoretical value of 0.19 ua/10^{-4} M GSH calculated from the Ilkovic equation (eqn. 5), using the diffusion coefficient value for GSH (51).

Thus, the above reasonings justify the the use of factor obtained with ATCH and in our experience, since the results are same with ATCH and GSH (provided one can get good GSH), it is more convenient to use ATCH than GSH.

Polarographic Equipment and Experimental Procedure.

A Metrohm Polarecord, model E261, with a maximum sensitivity of 0.025 ua full scale was employed in conjunction with a Metrohm polarographic stand, E354. The instrumental set up permitted both standard and rapid drop polarography.

A water jacketed micro reaction vessel with a capacity of 5 ml. connected to a thermostat maintained at 25.0 ± 0.1°C, was used for all polarographic measurements. All of the experimental measurements associated with ACHE-catalyzed hydrolysis of ATCH with and without inhibitors were made under the following conditions:

1.	Reaction volume	5 ml.
2.	Temperature	25.0 ± 0.1°C
3.	Applied potential	-0.05 V vs Ag/AgCl
4.	Current sensitivity	Variable
5.	Damping	3 or 4
6.	Chart speed	Slow
7.	Supporting electrolyte	Phosphate buffer, pH 7.6 (Appendix "A")
8.	Maximum suppressor	0.01% Gelatin
9.	Rapid drop	0.233 sec./drop
10.	Deaeration	With nitrogen for 8-10 minutes.

In all kinetic experiments an initial volume of 4.5 ml., made up of buffer solution, substrate solution, and inhibitor in case of inhibition study, was degassed with nitrogen for 8-10 minutes. The reaction was then started by adding 0.5 ml. of enzyme solution, giving a total reaction volume of 5.0 ml. Time measurements, made by calibrating the chart speed, were started after adding half of the enzyme solution. (36).

The conversion of initial rates in ua/min into umoles TCH/min was made according to the expression:

$$\text{umoles TCH/min} = \frac{\text{ua/min} \times 5.00 \pm 0.05 \times 10^{6}}{1000 \times 0.203 \pm 0.009 \text{ ua}/10^{-4} \text{ M TCH}}$$

Spectrophotometric Method. (38).

The indicator phenol red (pK = 7.9) is used along with a phosphate - barbital buffer of pH 8.0 to detect the pH changes due to acetic acid produced in the ACHE catalyzed hydrolysis of ATCH.(scheme 7). The acid dissociation constants of phenol red and barbital are within 0.1 pH units of each other. Therefore, as the liberated acetic acid converts a stoichiometric amount of sodium barbital into free diethyl barbituric acid, a much smaller but proportionate amount of alkaline phenol red is converted into the acid form. A spectrophotometric reading at 560 nm, in which only the alkaline form is measured, becomes therefore a direct measure of the enzyme activity.

The response of this system to acetic acid standards is linear to approximately 3.5×10^{-4} M (37). Since the initial rates were measured, the amount of acetate produced during the hydrolysis of ATCH did not exceed 3.5×10^{-4} M for any of the substrate concentrations used.

All experiments were carried out on a Cary Model 14 recording spectrophotometer with thermostated cell holders maintained at 25.00 ± 0.02°C and with the following instrumental and experimental parameters:

1. Wavelength	560 nm.
2. Slit height	20 mm.
3. Cell length	1 cm.
4. Temperature	25.00 ± 0.02°C
5. Reaction medium	Phosphate-Barbital buffer, pH 8.0 - with 0.03 ml. of 0.01% phenol red. (Appendix "A")
6. Reaction volume	5 ml.

The kinetic experiments were performed in the following way: In a small beaker, an initial volume of 4.5 ml made up of buffer, indicator, substrate and inhibitor, in case of inhibition experiments, was taken and to this 0.5 ml of enzyme solution was added to start the reaction. Mixing was ensured by blowing out the enzyme solution and by shaking the beaker. The reaction mixture was then immediately transferred to a 1 cm. cell and the kinetic measurements were started. This took

about 10 - 15 seconds and therefore reaction rates were measured 10 - 15 seconds after the addition of the enzyme solution.

All solutions were protected from the atmospheric carbon dioxide by storing under nitrogen and were pre - equilibriated at 25°C. Initial rates in the presence or absence of the inhibitor were calculated, as discussed previously, from the absorbance-time curves as absorbance units/min.

Materials.

Acetylcholinesterase. The enzyme preparation used was bovine erythrocyte acetylcholinesterase supplied by Sigma Chemical Co., St. Louis: Lot No. 61C-2350, with an activity of 3.2 units/mg solid and a specific activity of 8.6 units/mg protein. Lot No. 23C-1120, with an activity of 3.1 units/mg solid and a specific activity of 8.6 units/mg protein.

Since all kinetic experiments were performed at a constant enzyme concentration, the enzyme stock solutions were prepared as units/ml using the relationship 3.2 or 3.1 units/mg solid as reported by the supplier and activity was not determined under the experimental conditions used in this study. One unit of enzyme is defined as the amount of enzyme that will hydrolyze 1.0 umole of ACH to choline and acetate per minute at pH 8.0 at 37°C.

Acetylthiocholine. Acetylthiocholine chloride (M.W.201.7), the only

substrate used in this study, was supplied by Sigma Chemical Co., St.Louis. This was stored in a dessicator below $0^{\circ}C$.

Eserine. The inhibitor Eserine (Physostigmine) was also supplied by the Sigma Chemical Co., Eserine solutions are light sensitive and therefore were stored in dark bottles.

Glutathione. Reduced Glutathione was supplied by General Biochemicals, Lot 44613, and was stored below $0^{\circ}C$.

The acetylenic quaternary nitrogen compound (IV, Introduction) was kindly provided by Dr. M.D.Simon, Faculty of Pharmacy, University of Montreal.

N.B. All the materials were used as supplied.

RESULTS AND DISCUSSION

Part I. Effect Of Substrate Concentration.

The effect of substrate concentration on the reaction rate was studied at three different enzyme concentrations in order to choose an appropriate enzyme concentration to be used in inhibition studies and also to test the applicability of Michaelis-Menten kinetics.

Although in most cases the rate is proportional to enzyme concentration, as is assumed in Michaelis - Menten formulation, and in fact the usual expressions for inhibition kinetics are derived on this basis, there are several reasons for non-linearity. Factors which can give rise to non-linear relationship are as follows (41): the presence of a reversible inhibitor in the enzyme preparation, velocity measurements made as amount of product in a given time instead of initial rates, slow response of the instrument used for velocity measurements, using an excess of enzyme, etc. Therefore it is of importance to use an enzyme concentration where the reaction rate is linearly related to it, since non-linearity can easily alter inhibition behaviour. This linear relationship can be checked by plotting initial rates obtained at different enzyme concentrations versus enzyme concentration at a constant substrate concentration.

To test whether or not the system behaves according to the Michaelis-Menten formulation (eqn.1) , Km

and Vmax values should be calculated at different enzyme concentrations. According to this formulation the Km is independent of, and the Vmax is proportional to, the enzyme concentration.

It is known that Acetylcholinesterase is inhibited by an excess of substrate (42). In a system showing the substrate inhibition the Km and Vmax values are calculated from the substrate concentrations showing no inhibition (43).

The three enzyme concentrations used in this study were: 0.31 ± 0.01, 0.51 ± 0.02, and 0.81 ± 0.03 units of ACHE per 5 ml. of the reaction mixture. Tables I - III show the substrate concentration-velocity data obtained at each of the enzyme concentration. Each value represents an average of at least three measurements. Data for individual runs are listed in Appendix "C".

The calculation of Km and Vmax values were made using only the lower substrate concentrations and employing the weighted non-linear regression analysis of Wilkinson (44).

Table I. Substrate concentration-rate data for ATCH hydrolysis.

ACHE = 0.31 ± 0.01 units per 5 ml of the reaction mixture.

ATCH Moles liter^{-1}	v, umoles TCH/min
$6.1 \pm 0.1 \times 10^{-5}$	0.030 ± 0.006
$1.10 \pm 0.03 \times 10^{-4}$	0.044 ± 0.002
$2.20 \pm 0.06 \times 10^{-4}$	0.074 ± 0.005
$5.8 \pm 0.1 \times 10^{-4}$	0.118 ± 0.007
$1.17 \pm 0.02 \times 10^{-3}$	0.14 ± 0.01
[a] $1.99 \pm 0.05 \times 10^{-3}$	0.14 ± 0.01
[a] $3.98 \pm 0.09 \times 10^{-3}$	0.14 ± 0.01

Km and Vmax values calculated from the data of Table I were:

$$Km = 3.2 \pm 0.2^{*} \times 10^{-4} \text{ M}$$

$$Vmax = 0.179 \pm 0.004^{*} \text{ umole TCH/min}$$

a - values not includede in the calculation of Km and Vmax.
* - standard deviation.

Table II. Substrate concentration-rate data for ATCH hydrolysis.

ACHE = 0.51 ± 0.02 units per 5 ml of the reaction mixture.

ATCH Moles liter^{-1}	v, umoles TCH/min
$9.9 \pm 0.2 \times 10^{-5}$	0.076 ± 0.008
$1.98 \pm 0.05 \times 10^{-4}$	0.130 ± 0.008
$3.97 \pm 0.09 \times 10^{-4}$	0.18 ± 0.02
$6.0 \pm 0.1 \times 10^{-4}$	0.22 ± 0.02
$7.9 \pm 0.1 \times 10^{-4}$	0.23 ± 0.02
[a] $9.9 \pm 0.2 \times 10^{-4}$	0.23 ± 0.02
[a] $1.98 \pm 0.05 \times 10^{-3}$	0.24 ± 0.01
[a] $3.96 \pm 0.09 \times 10^{-3}$	0.24 ± 0.01

Km and Vmax values calculated from the data of Table II were:

$$Km = 3.1 \pm 0.3^{*} \times 10^{-4} \text{ M}$$

$$Vmax = 0.32 \pm 0.01^{*} \text{ umole TCH/min}$$

a - values not included in the calculation of Km and Vmax.
* - standard deviation.

Table III. Substrate concentration-rate data for ATCH hydrolysis.

ACHE = 0.81 ± 0.03 units per 5 ml of the reaction mixture.

ATCH Moles liter^{-1}	v, umoles TCH/min
$1.05 \pm 0.03 \times 10^{-4}$	0.12 ± 0.01
$2.10 \pm 0.06 \times 10^{-4}$	0.20 ± 0.02
$5.2 \pm 0.1 \times 10^{-4}$	0.31 ± 0.02
$1.03 \pm 0.03 \times 10^{-3}$	0.41 ± 0.02
[a] $2.00 \pm 0.05 \times 10^{-3}$	0.42 ± 0.02
[a] $4.0 \pm 0.1 \times 10^{-3}$	0.41 ± 0.02

Km and Vmax values calculated from the data of Table III were:

$Km = 3.3 \pm 0.5^{*} \times 10^{-4}$ M

$Vmax = 0.50 \pm 0.03^{*}$ umole TCH/min

a - values not included in the calculation of Km and Vmax.
* - standard deviation.

The data of Tables I - III are plotted in Fig. 3, the rate - substrate concentration curves. The plateau of these curves represents the experimental maximum rate, v_o. From the Michaelis-Menten equation (eqn. 1) it is clear that maximum rate can only be observed when $S \gg Km$, therefore the maximum rate observed in this case is due to the fact that ACHE is inhibited by an excess of substrate.

Fig. 4 shows the Lineweaver-Burke plot at each enzyme concentration. The rise in the curves as they approach 1/v axis is due to substrate inhibition. (8). In Table IV below the Km, Vmax, and v_o values are listed.

Table IV. Vmax, v_o, and Km values for ATCH hydrolysis at different concentrations of ACHE.

ACHE units	Vmax umole TCH/min	v_o umole TCH/min	Km x 10^4 moles liter^{-1}
0.31 ± 0.01	0.179 ± 0.004	0.14 ± 0.01	3.2 ± 0.2
0.51 ± 0.02	0.32 ± 0.01	0.24 ± 0.01	3.1 ± 0.3
0.81 ± 0.03	0.50 ± 0.03	0.42 ± 0.02	3.3 ± 0.5

Table IV shows that Km is independent of enzyme concentration as is expected from the Michaelis-Menten equation (eqn. 1). The theoretical maximum rate, Vmax, at different enzyme concentrations is plotted in Fig. 5 and shows a linear dependence on the enzyme concentration.

Fig. 3. Effect of substrate concentration on the reaction rate.

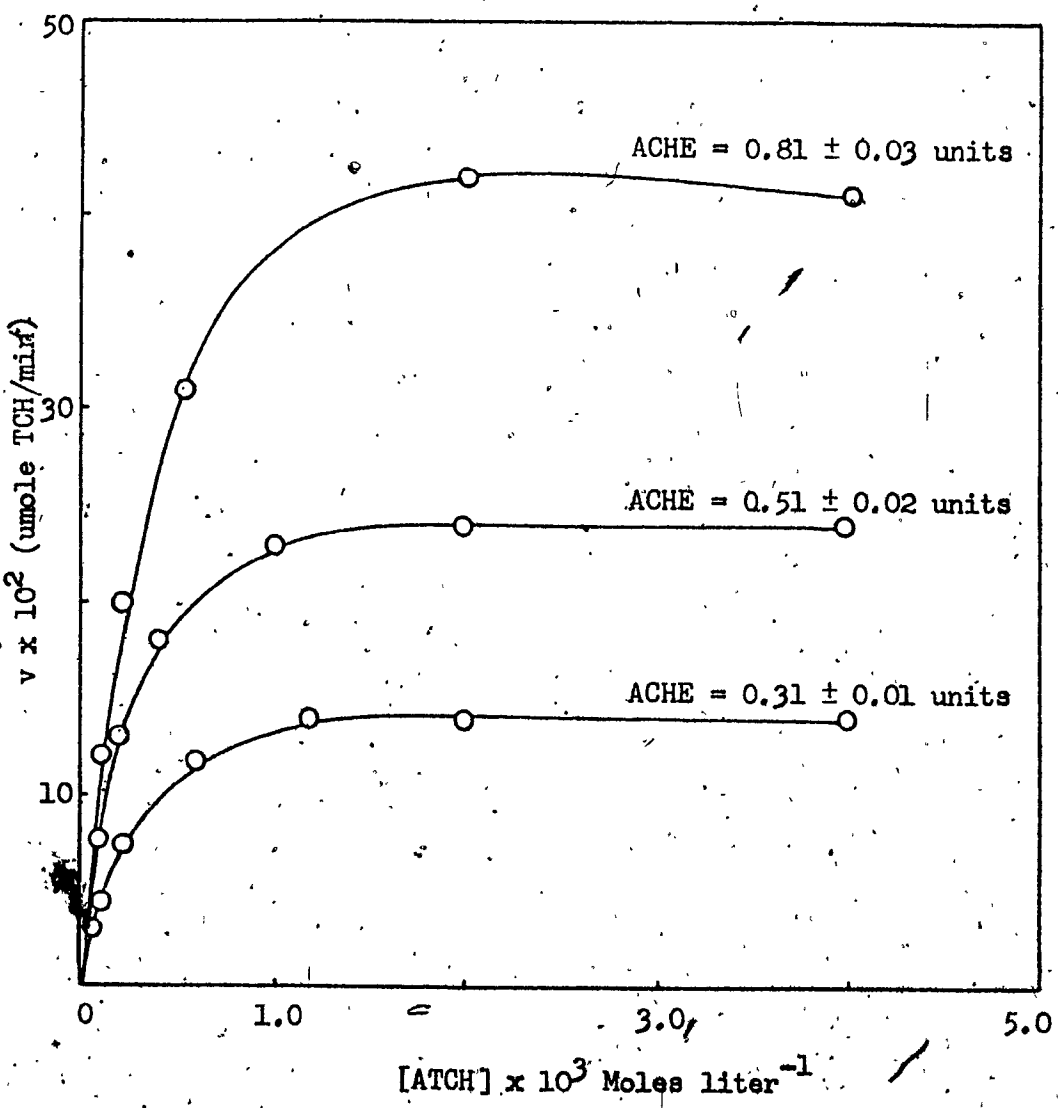


Fig. 4. Lineweaver-Burke plot of ACHE-catalyzed hydrolysis of ATCH at different concentrations of ACHE.

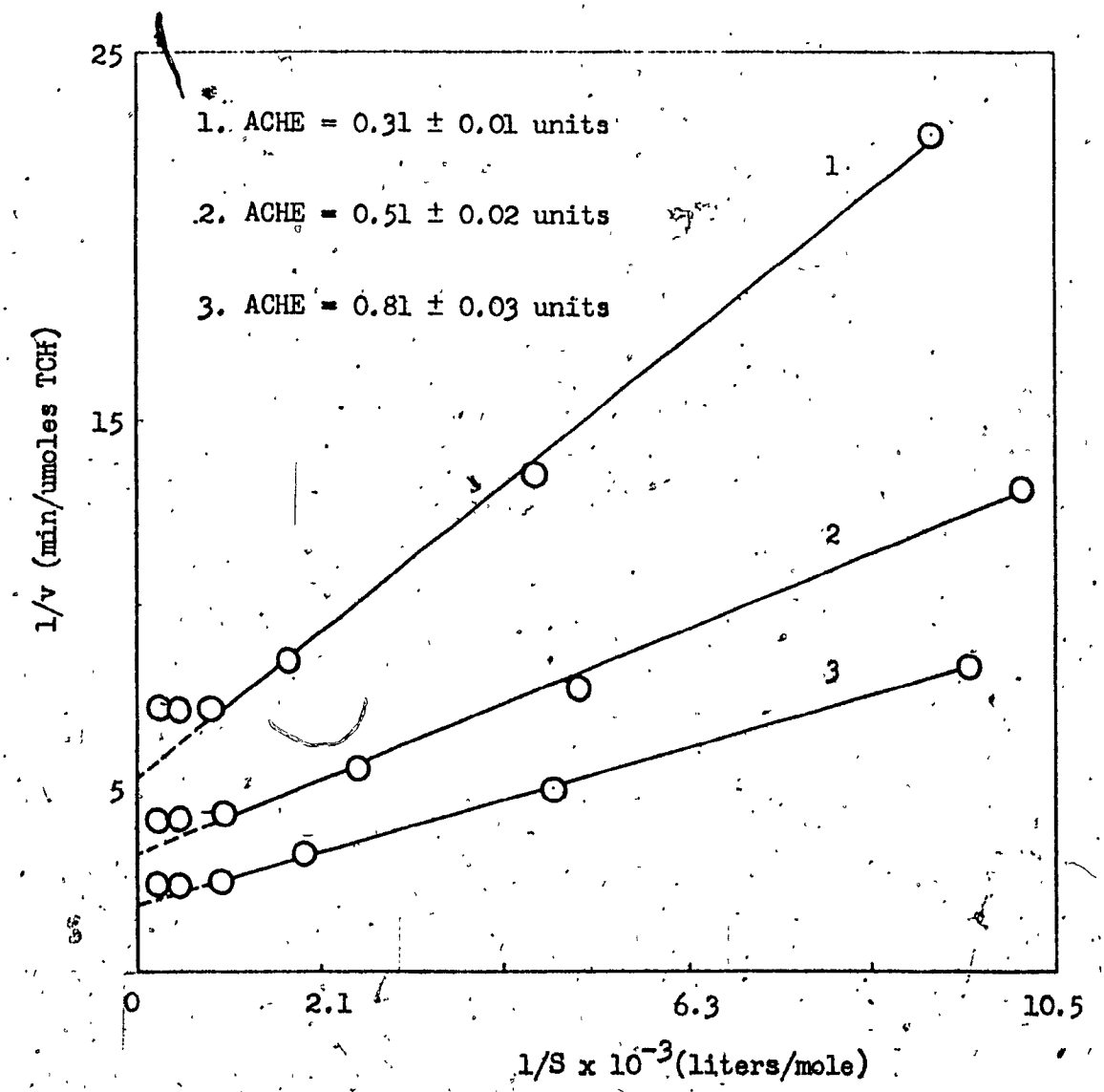
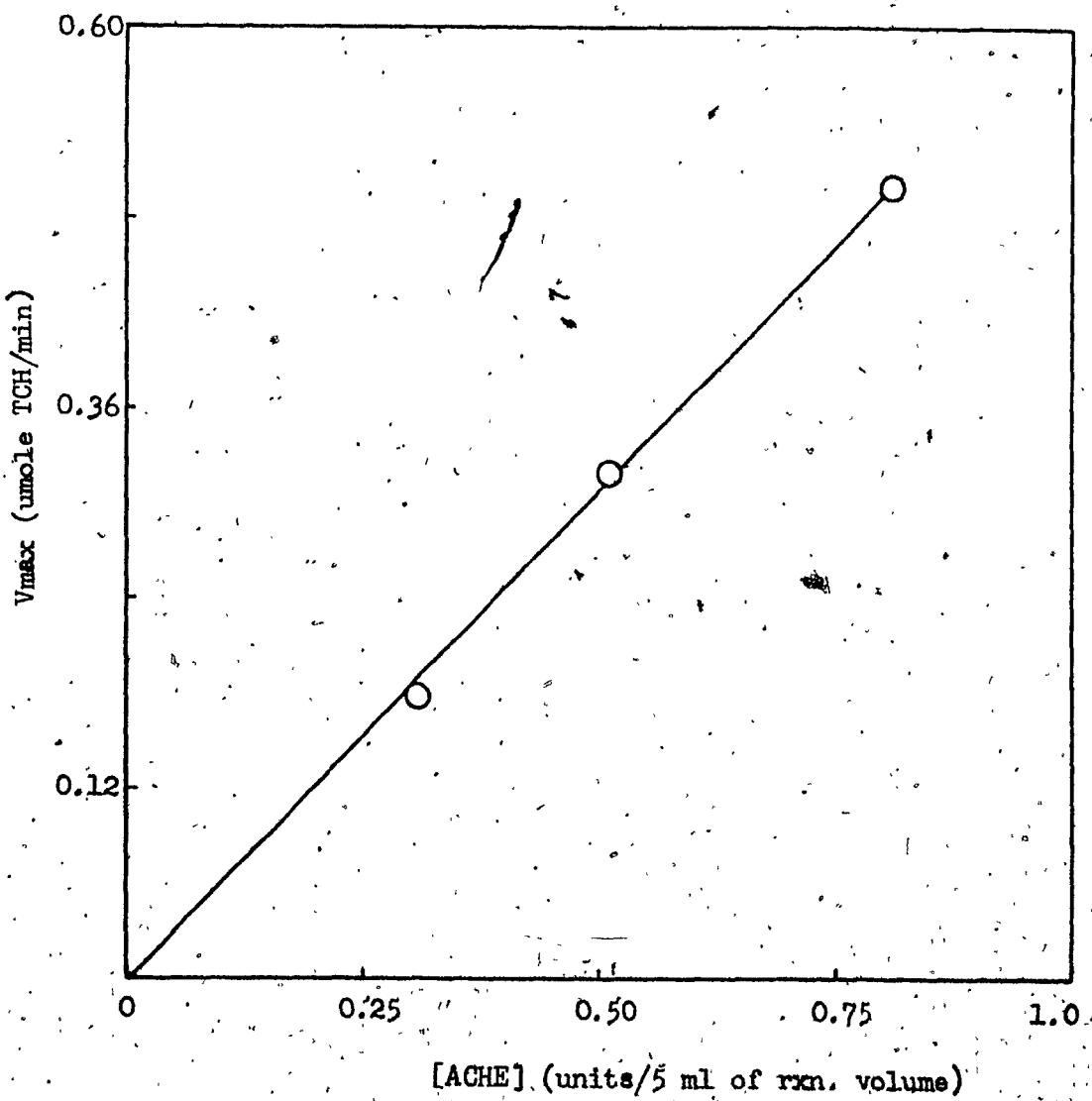


Fig. 5. Effect of Acetylcholinesterase concentration on Vmax.



Since Vmax is a theoretical value, the linear relationship was also established by plotting the experimental rate, v, obtained at a lower substrate concentration ($1.05 \pm 0.03 \times 10^{-4}$ M ATCH) against the enzyme concentration (Fig. 6). In Fig. 7, the experimental maximum rate, v_o, is plotted against the enzyme concentration. Though this plot is linear, it does not pass through the origin implying that some enzyme is not available for the catalysis. This can be explained from the fact, that, since the experimental maximum rate is observed due to the substrate inhibition, therefore there is some enzyme, which is inhibited by the substrate, and thus not available for the catlysis.

The Km value obtained in this study (see Table IV) is higher than the reported value of 1.31×10^{-4} M (22) and 1.40×10^{-4} M (16) for ATCH using other methods for measuring rates. The high value obtained in this study is probably due to the high ionic strength used. High ionic strengths decrease the binding of the substrates to the enzyme and thus result in high Km values. The effects of ionic strength on ACHE-catalyzed hydrolysis and its inhibition will be discussed in detail later on.

A Km value of 3.23×10^{-4} M for ATCH, using ATCH iodide, was obtained by Ridgway and Mark jr.(52). These workers used the polarographic method; the ionic strength used was 0.05. The high Km value obtained by thses workers, using low ionic strength, is probably due to the fact that they used an iodide salt. We have found that iodide interferes in the determination of TCH

because it gives an anodic wave in the same potential region as does the TCH wave. The effect of iodide will be discussed in detail later.

In summary, the "Effect of substrate concentration" study shows the following:

1. The Km value is independent of, and the Vmax is proportional to, the enzyme concentration, thus showing that the system follows the Michaelis-Menten kinetics.

2. No lowering of rates, due to the substrate inhibition, occurs up to approximately 4×10^{-3} M ATCH.

3. The rate is linearly dependent on ACHE concentration up to 0.8 units of ACHE per 5 ml of the reaction mixture. Therefore any ACHE concentration in this range can be used in inhibition studies.

4. The experimental maximum rate, v_o, is observed due to the fact that ACHE is inhibited by an excess of substrate. The v_o vs ACHE concentration plot does not pass through the origin because of substrate inhibition.

Fig. 6. Effect of Acetylcholinesterase concentration on the reaction velocity, v, at $1.05 \pm 0.03 \times 10^{-4}$ M ATCH.

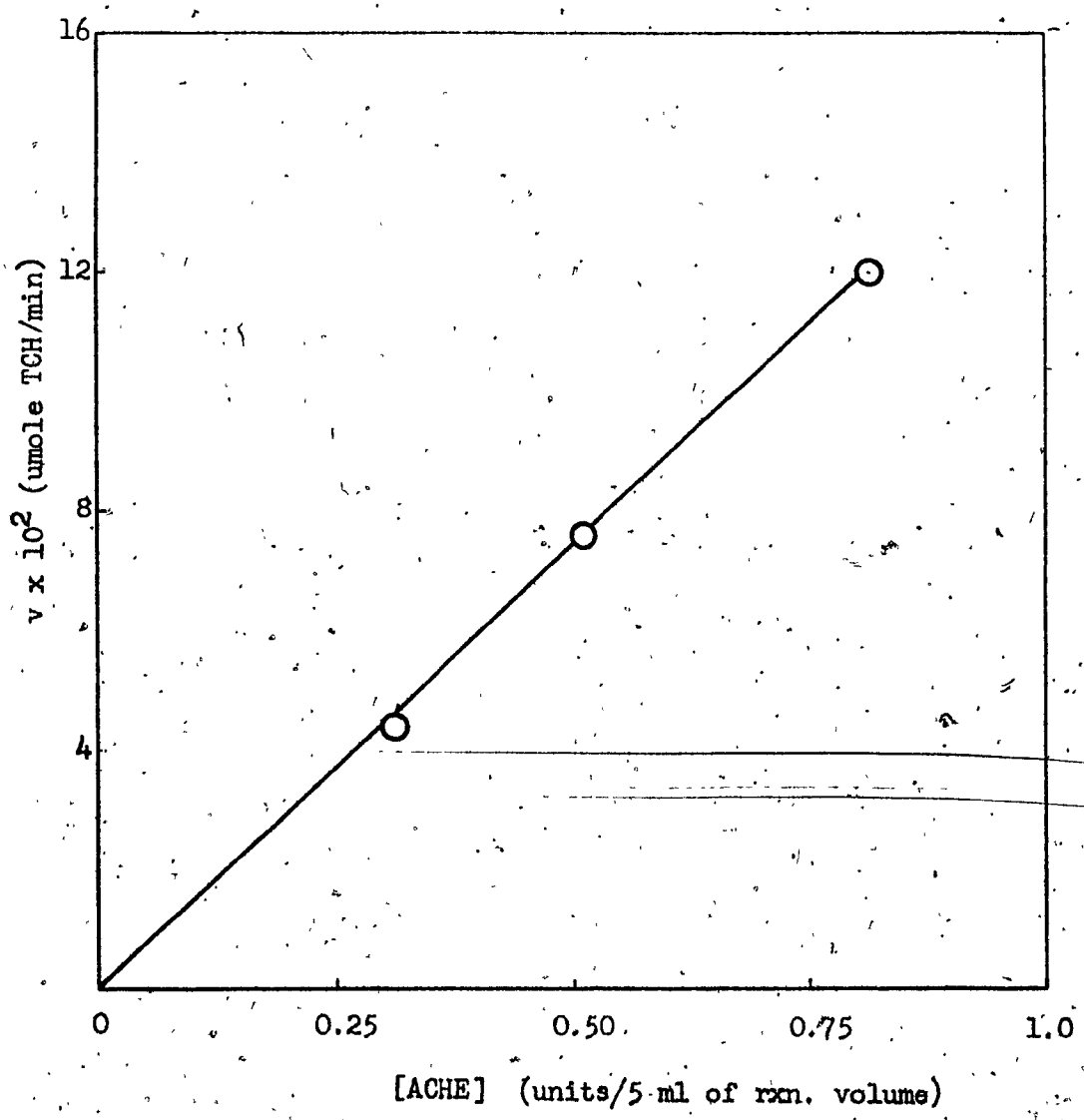
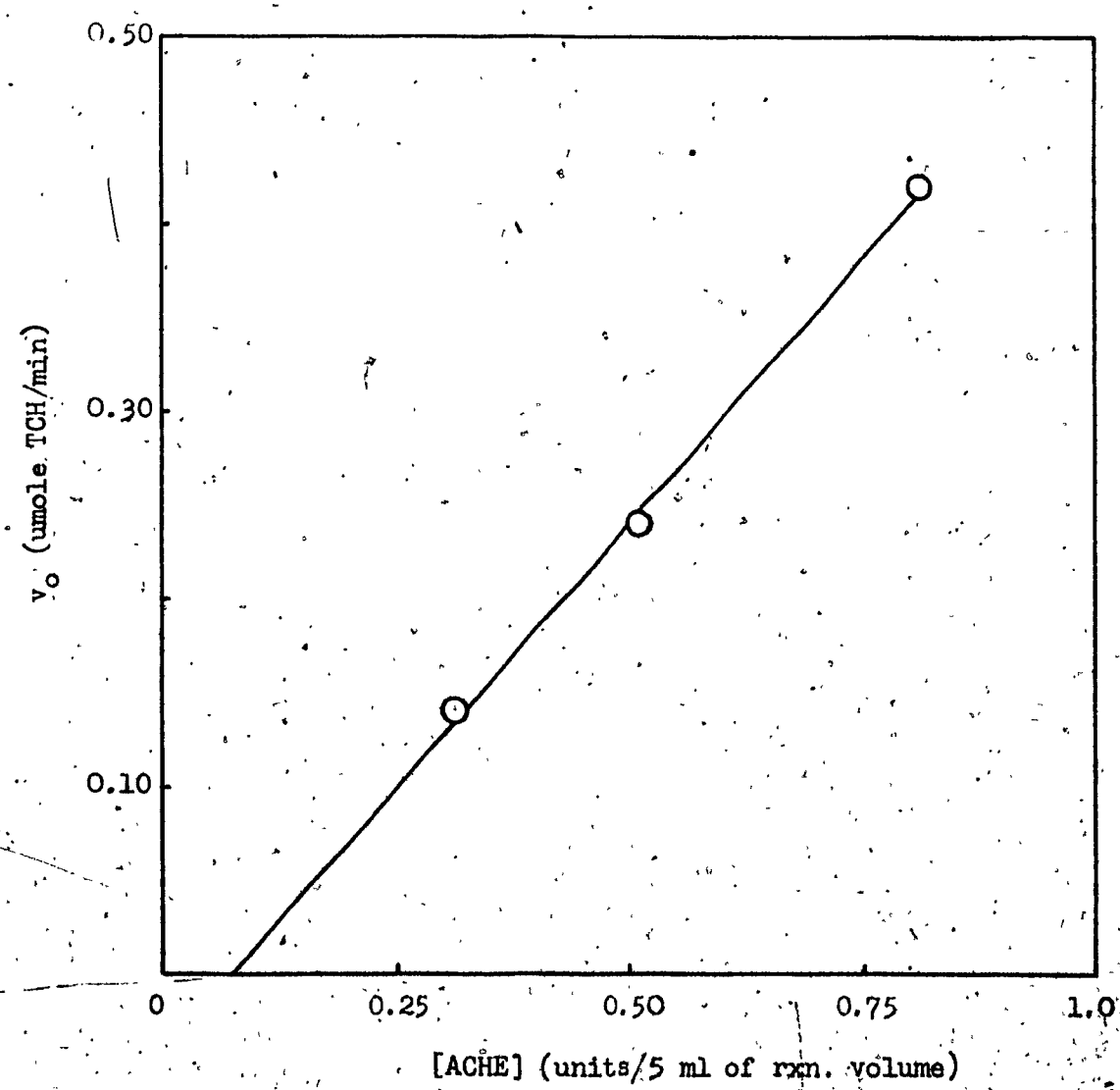


Fig. 7. Effect of Acetylcholinesterase concentration on experimental maximum rate, v_o.



Part II. Inhibition of ACHE by eserine - Polarographic Method.

Inhibition of ACHE by eserine was studied at zero enzyme-eserine incubation time. This was done at three different concentrations of eserine and the effect of substrate concentration was studied at each of the eserine concentrations. Thses experiments were carried out in a buffer of pH 7.6, ionic strength 1.0, and with 0.01% gelatin (see appendix "A"). In each run about 0.5 units of ACHE per 5 ml of the reaction mixture was used.

The inhibition data are listed in Table V and the data for individual runs are listed in appendix "D". As can be seen, at each eserine concentration, the percent inhibition decreases as the substrate concentration increases, as would be expected for simple competitive inhibition. The Km and Vmax values were calculated at each of the eserine concentrations and are listed in Table VI. This data shows that the Km value increases with an increase in the concentration of eserine whereas Vmax stays constant. This is in accord with the concept of competitive inhibition, where an inhibitor competes with the substrate for the enzyme, thus increasing Km, but does not interfere with the breakdown of the enzyme-substrate complex and thus has no effect on Vmax.

Thus the data of Tables V and VI shows that eserine inhibits competitively under the conditions of zero incubation time. This means that under these conditions no carbamylation of the enzyme takes place or in other words the

Table V. Inhibibtion by eserine at zero incubation time using polarographic method.

ATCH (moles liter^{-1}) x 10^3	Eserine (moles liter^{-1} x 10^6) 0	1.10 ± 0.02		2.10 ± 0.04		4.21 ± 0.07	
	v	v_i	% inhibition	v_i	% inhibition	v_i	% inhibition
0.222 ± 0.006	0.13 ± 0.01	0.10 ± 0.01	23	0.084 ±0.009	35	0.06 ± 0.01	54
0.50 ± 0.01	0.20 ± 0.02	0.16 ± 0.02	20	0.14 ± 0.01	30	0.12 ± 0.01	40
0.75 ± 0.02	0.24 ± 0.02	0.21 ± 0.02	12	0.18 ± 0.01	25	0.16 ± 0.01	33
1.01 ± 0.02	0.25 ± 0.02	0.23 ± 0.02	8	0.21 ± 0.01	16	0.18 ± 0.01	28
[a] 1.99 ± 0.05	0.25 ± 0.02	0.23 ± 0.01	8	0.22 ± 0.01	12	0.20 ± 0.02	20
[a] 3.98 ± 0.09	0.25 ± 0.02	0.25 ± 0.02	0	0.24 ± 0.01	4	0.23 ± 0.01	8

v - velocity in the absence of eserine in umoles TCH/min.
v_i - velocity in the presence of eserine in umoles TCH/min.
a - concentrations not used in the calculation of Km, Vmax, and K_i values.

substrate protects the enzyme from carbamylation as stated by Wilson (23).

Table VI. Effect of eserine on Km and Vmax.

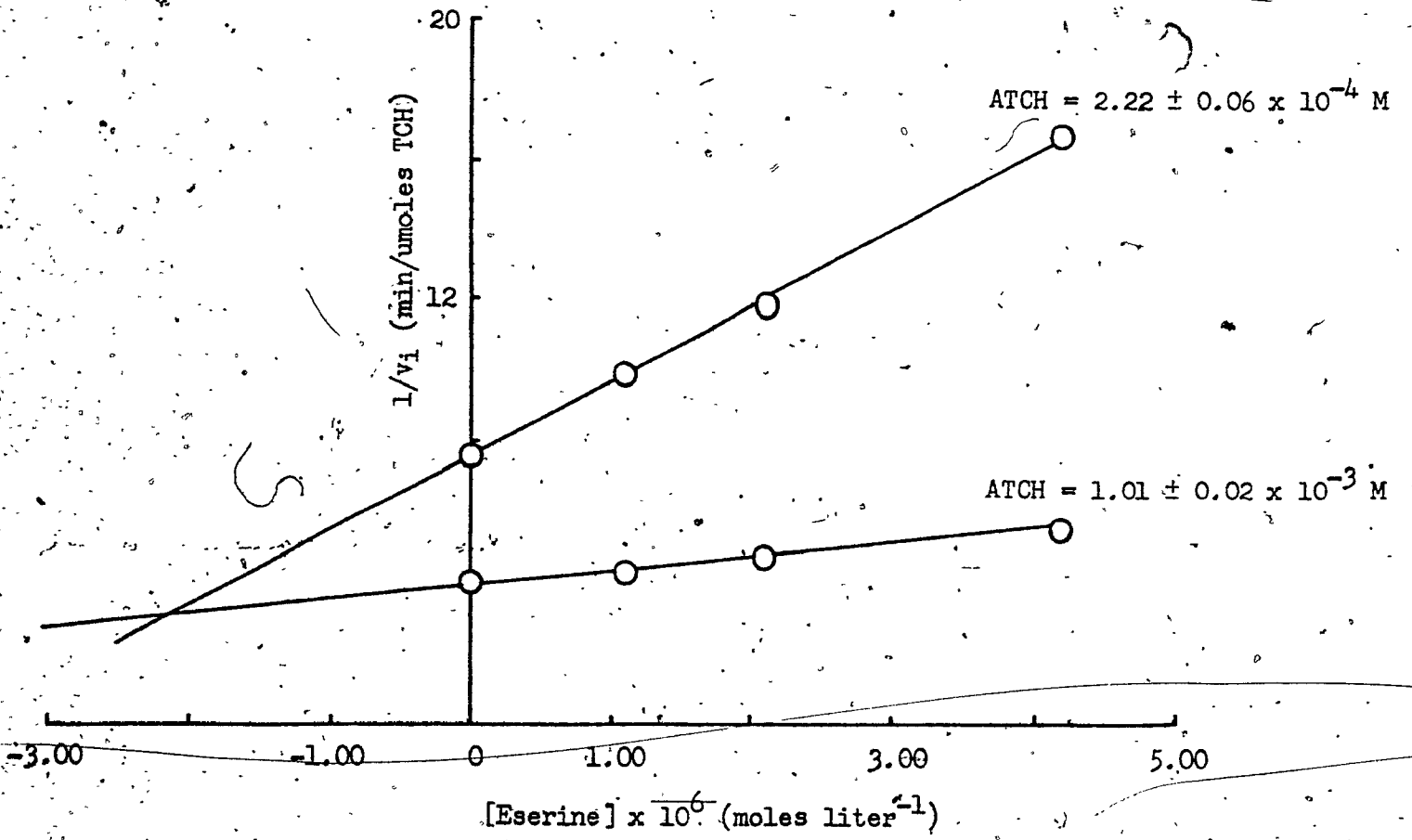
Eserine moles liter^{-1} x10^6	Km moles liter^{-1} x 10^4	Vmax umoles TCH/min
0	3.6 ± 0.5	0.35 ± 0.02
1.10 ± 0.02	6 ± 1	0.38 ± 0.03
2.10 ± 0.04	8.1 ± 0.9	0.38 ± 0.02
4.21 ± 0.07	11 ± 2	0.39 ± 0.05

N.B. Km and Vmax values were calculated as discussed before.

The value of the inhibitor constant, K_i, was calculated according to the method of Dixon (47) by plotting the reciprocal of velocity versus inhibitor concentration at different substrate concentrations. The point on the abscissa corresponding to the intersection of these lines gives the value equal to $-K_i$. Fig. 8 shows such a plot at two different concentrations of ATCH.

An average value of K_i was calculated from the intersection of 1/v vs I plots at four different

Fig. 8. $1/v_i$ vs I plot for the inhibition of ACHE by eserine at two different concentrations of ATCH.



concentrations of ATCH. This resulted in six intersection points, each giving a K_i value. These are listed in Table VII below.

Table VII. K_i values determined from the $1/v_i$ vs I plots at different concentrations of ATCH.

ATCH concentration used for $1/v_i$ vs I plots (moles/liter)	$K_i \times 10^6$ (moles/liter)
$2.22 \pm 0.06 \times 10^{-4}$ and $5.0 \pm 0.1 \times 10^{-4}$	1.76
$2.22 \pm 0.06 \times 10^{-4}$ and $7.5 \pm 0.2 \times 10^{-4}$	2.07
$2.22 \pm 0.06 \times 10^{-4}$ and $1.01 \pm 0.02 \times 10^{-3}$	2.08
$5.0 \pm 0.1 \times 10^{-4}$ and $7.5 \pm 0.2 \times 10^{-4}$	3.60
$5.0 \pm 0.1 \times 10^{-4}$ and $1.01 \pm 0.02 \times 10^{-3}$	3.18
$7.5 \pm 0.2 \times 10^{-4}$ and $1.01 \pm 0.02 \times 10^{-3}$	2.25

From the K_i values listed in Table VII, an average value of $2.5 \pm 0.4 \times 10^{-6}$ M was secured. It may be helpful to point out here that the inhibitor constant, K_i, is the dissociation constant of the EI complex into free enzyme and inhibitor (scheme 9), therefore, the lower the K_i value the greater the effectiveness of an inhibitor.

Only the lower substrate concentrations were used in the calculation of the inhibitor constant because of substrate inhibition at higher concentrations.

Spectrophotometric method. Inhibition of ACHE by eserine was also studied by spectrophotometric method. These experiments were carried out in a buffer solution of pH 8.0. The ionic strength of this solution was approximately 0.15 (see appendix "A"). The concentration of ACHE used in these experiments was around 0.5 units per 5 ml of the reaction mixture. These experiments were also carried out at zero enzyme-eserine incubation time.

The inhibition data are listed in Table VIII and the data for individual runs are listed in appendix "D".

The data of Table VIII shows that the percent inhibition decreases as the substrate concentration increases implying the competitive inhibition. The Km and Vmax values were also calculated and are listed in Table IX. The substrate concentrations showing inhibition were not used in the calculation of Km and Vmax values. The data of Table IX shows that the Km value increases with the increase in the concentration of eserine while the Vmax is unaffected. This is what to be expected from the inhibitors acting competitively.

The Km value of $1.0 \pm 0.3 \times 10^{-4}$ M, in the absence of eserine, agrees well with the reported Km values of 1.31×10^{-4} M (22) and 1.40×10^{-4} M (16) for ATCH. A value of $3.3 \pm 0.4 \times 10^{-4}$ M for Km was calculated from the data of Stein and Lewis (31). The high value of Km from the data of these workers is probably due to the high ionic strengths used by them.

Table VIII. Inhibition by eserine at zero incubation time using spectrophotometric method.

ATCH (moles liter^{-1}) x 10^3	Eserine (moles liter^{-1} x 10^6) 0		1.08 ± 0.02		2.08 ± 0.03		4.17 ± 0.06	
	v	v_i	% inhibition	v_i	% inhibition	v_i	% inhibition
0.214 ± 0.006	0.038±0.000	0.028±0.000	26	0.020±0.001	47	0.014±0.000	63
0.51 ± 0.01	0.044±0.001	0.035±0.000	20	0.029±0.001	34	0.020±0.000	55
1.01 ± 0.01	0.051±0.000	0.046±0.000	10	0.040±0.002	22	0.034±0.001	34
[a]2.00 ± 0.05	0.047±0.001	0.040±0.001	14	0.036±0.002	23	0.033±0.001	30
[a]4.00 ± 0.09	0.044±0.000	0.041±0.000	7	0.038±0.001	14	0.037±0.000	16

v - velocity in the absence of eserine in absorbance units/min.
v_i - velocity in the presence of eserine in absorbance units/min.
a - concentrations not used in the calculation of Km, Vmax, and K_i values.

Table IX. Effect of eserine on Km and Vmax.

Eserine moles liter^{-1} x 10^6	Km moles liter^{-1} x 10^4	Vmax Abs. units/min
0	1.0 ± 0.3	0.055 ± 0.003
1.08 ± 0.02	2 ± 1	0.054 ± 0.007
2.08 ± 0.03	4 ± 1	0.055 ± 0.007
4.17 ± 0.06	9 ± 5	0.06 ± 0.02

The large deviations in the Km and Vmax values are due to the fact that only three concentrations of ATCH were used in their calculations. Nevertheless, these values show the competitive nature of the inhibition.

The K_i value was calculated from the intersection of $1/v_i$ versus I plots at different concentrations of ATCH. Fig. 9 shows such a plot at two different concentrations of ATCH. An average value of K_i was calculated from the intersection of $1/v_i$ vs I plots at three different concentrations of ATCH. The values are listed in Table X.

Fig. 9. $1/v_i$ vs I plot for the inhibition of ACHE by eserine at two different concentrations of ATCH.

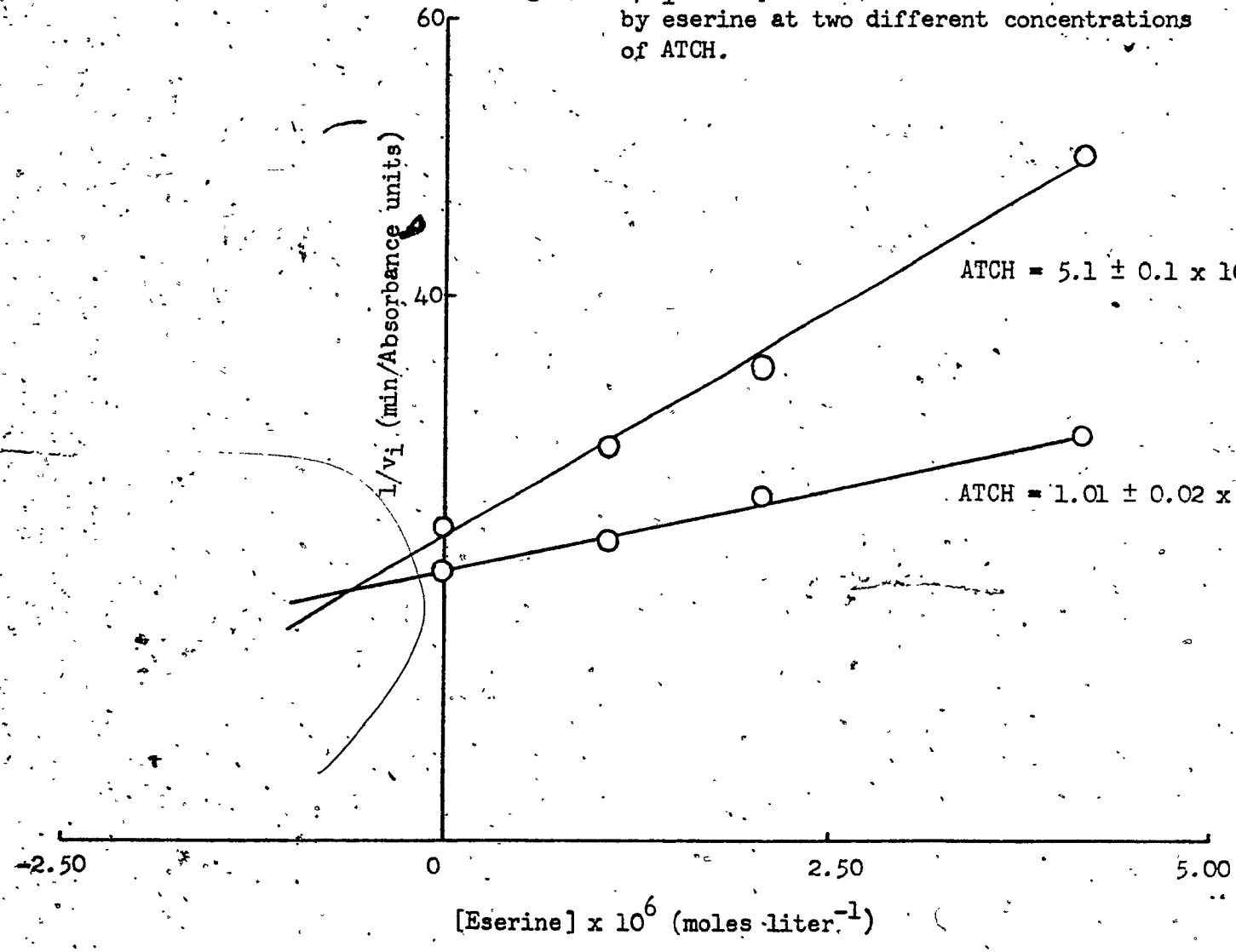


Table X. K_i values determined from the $1/v_i$ vs I plots at different concentrations of ATCH.

ATCH concentration used for $1/v_i$ vs I plots (moles/liter)	$K_i \times 10^7$ (moles/liter)
$2.14 \pm 0.06 \times 10^{-4}$ and $5.1 \pm 0.1 \times 10^{-4}$	8.50*
$2.14 \pm 0.06 \times 10^{-4}$ and $1.01 \pm 0.02 \times 10^{-3}$	5.53
$5.1 \pm 0.1 \times 10^{-4}$ and $1.01 \pm 0.02 \times 10^{-3}$	5.54

* - value rejected by "Q" test.

The average K_i value secured was $5.54 \pm 0.00 \times 10^{-7}$ M.

Comparison of data secured by polarographic and spectrometric method.

Qualitatively the two methods gave the similar results. However, an inspection of Tables V and VIII shows the following features:

1. The data of Table VIII shows substrate inhibition at high concentrations of ATCH while the Table V shows only maximum rate, at comparable concentrations of ATCH.

2. The percentage inhibition obtained by the spectrophotometric method, at comparable concentrations of eserine, is more than that obtained by the polarographic method.

3. The difference in the K_i values obtained by the two methods.

The above differences can be explained from the difference in the ionic strengths of the reaction media employed in these methods.

Since the active site of ACHE is negatively charged, the Na^+ ions (sodium nitrate was used for adjusting ionic strength of the buffer solution used in the polarographic method) compete with the substrate and with the positively charged inhibitor molecules for this site. It was shown by Krupka (48) that the substrate molecules inhibit by combining with the anionic site of the acetyl enzyme and blocking deacetylation. The competition of sodium ions with the excess of substrate molecules for the acetyl enzyme probably protects it from inhibition by the excess of substrate and that is why inhibition by substrate has not been observed by the polarographic method in which buffer solution of ionic strength 1.0 was employed. In the spectrophotometric method, where ionic strength of the reaction medium was about 0.15, no such effects exist and the inhibition is observed around 2×10^{-3} M ATCH. It must be mentioned here that some preliminary experiments were conducted to determine at what concentration of the substrate, at ionic strength of 1.0, appreciable inhibition due to excess of substrate occurs. It was found that appreciable inhibition by substrate occurs around 6×10^{-3} M ATCH. Thus the net effect of higher ionic strengths is to shift the substrate concentration to higher values for comparable substrate

inhibition observed at lower ionic strengths. This effect has also been observed by Myers (49).

The K_i value is a measure of an inhibitors binding to the enzyme. Since the positively charged form of eserine is the enzyme inhibitor, high ionic strengths decrease this binding and this accounts for the low percent inhibitions and high K_i value , observed with the polarographic method. Similar effects of high ionic strengths on the enzyme inhibition have been observed in other enzymes involving charged groups on the active site (50).

The calculated K_i value of $2.5 \pm 0.4 \times 10^{-6}$ M with the polarographic method (ionic strength 1.0) and $5.54 \pm 0.00 \times 10^{-7}$ M with the spectrophotometric method (ionic strength 0.15) agrees well with the reported value of 3.1×10^{-6} M with an ionic strength of 0.5 (31) and 2.22×10^{-7} M with an ionic strength of 0.16 (25).

In summary, the inhibition of ACHE by eserine studies, using the polarographic and the spectrophotometric methods, shows the following:

1. That the eserine inhibits ACHE competitively when the substrate and inhibitor are added to the enzyme at the same time (i.e. under zero enzyme-eserine incubation time).

2. That the effect of high ionic strengths is to decrease the percent inhibition and to increase the K_i value.

3. That the polarographic and the spectrophotometric methods give similar results and the differences in percent inhibition and K_i values are may be due to the differences in ionic strengths.

4. The comparison of Ki value, calculated using a polarogrphic method, with the literature values shows that this method can be used for studying ACHE inhibition by compounds like eserine (i.e. carbamates).

Part III. Inhibition of ACHE by 1 -(4-dimethylamino-2-butynyloxy)-3-(2-propynyloxy) benzene methiodide (IV).

Inhibition of ACHE by this compound (referred as inhibitor or quaternary compound in the text) was studied polarographically. These experiments were carried out in a buffer of pH 7.6, ionic strength 0.5, and with 0.01% gelatin. Since ionic strength greatly affects the ACHE hydrolysis and inhibition, the ionic strength in this study was reduced to half of the previous value of 1.0. These experiments were also conducted with 0.5 units of ACHE per 5 ml of the reaction mixture.

It is known that the iodide gives an anodic wave with $E_{1/2}$ of -0.03 V vs S.C.E. (53). This inhibitor was an iodide salt and therefore gives an anodic wave as shown in Fig. 10. A comparison of this with TCH wave (Fig. 1) shows that the wave due to iodide is in the same potential range as the TCH wave. It may be noted that TCH wave starts at more negative potentials but nevertheless the useful potential range which can be used for following TCH production is also covered by the iodide wave.

Since the wave due to iodide does not change with time, it was thought that the rates can be followed by taking the current due to this wave as the base line and following the increase in current due to the production of TCH as before. The data of Table XI, which shows the effect of substrate concentration

Fig. 10. The polarographic wave due to iodide obtained with quaternary nitrogen compound (IV).

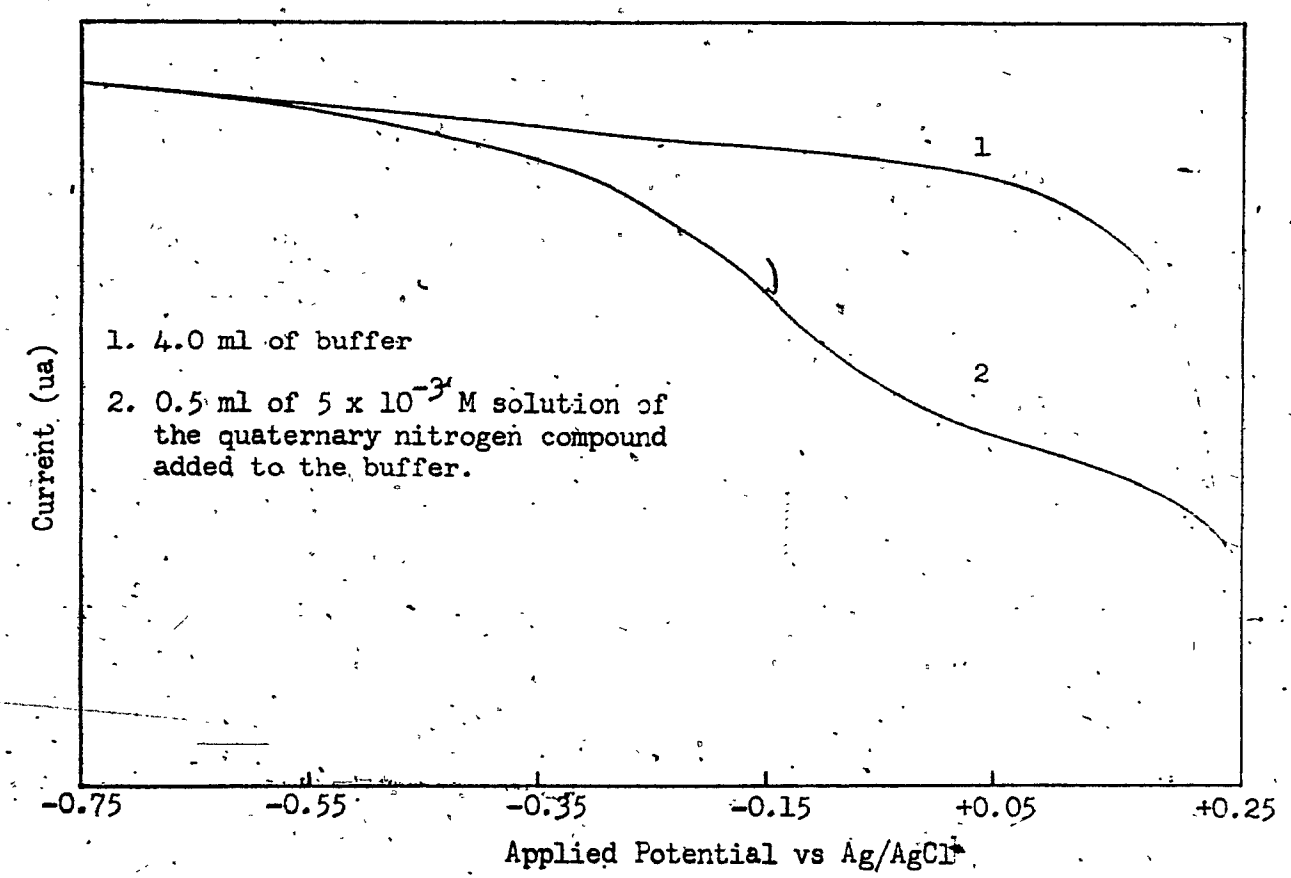


Table XI. Inhibition of AChE by the quaternary compound (IV) at zero incubation time using the polarographic method.

ATCH (moles/liter^{-1}) x 10^3	[I] x 10^4 (moles liter^{-1})							
	0	2.50 ± 0.07		5.0 ± 0.1		7.5 ± 0.1		
	v_o	v_i	% inhibition	v_i	% inhibition	v_i	% inhibition	
0.212 ± 0.006	0.18 ± 0.01	0.13 ± 0.01	28	0.086 ± 0.009	52	0.054 ± 0.007	70	
0.41 ± 0.01	0.27 ± 0.01	0.21 ± 0.01	22	0.14 ± 0.01	48	0.11 ± 0.01	59	
0.62 ± 0.01	0.29 ± 0.01	0.23 ± 0.01	21	0.16 ± 0.01	45	0.13 ± 0.01	56	
0.83 ± 0.02	0.30 ± 0.02	0.23 ± 0.01	23	0.15 ± 0.01	50	0.11 ± 0.01	64	
1.24 ± 0.03	0.33 ± 0.02	0.25 ± 0.01	24	0.17 ± 0.01	49	0.12 ± 0.01*	64	

v - velocity in the absence of inhibitor, in umoles TCH/min
v_i - velocity in the presence of inhibitor in umoles TCH/min
* - single measurement

on percent inhibition at three different concentrations of this inhibitor, was secured in this way. The data of this Table shows that the percent inhibition does not decrease by any appreciable amount as the substrate concentration increases at a given concentration of this inhibitor. This implies non-competitive inhibition.

But before any further conclusions could be drawn from this data, it was desired to test whether or not one can take the current due to the iodide wave as the base line. To this end, the effect of similar concentrations of potassium iodide on ATCH hydrolysis was studied and the data is listed in Table XII.

Table XII. Effect of Potassium Iodide on ATCH hydrolysis.

$[ATCH] = 6.3 \pm 0.1 \times 10^{-4}$ M

$[KI] \times 10^4$ moles/liter	rate, v, umoles TCH/min	contribution to % inhibition
0	0.24 ± 0.01	-
2.52 ± 0.09	0.21 ± 0.01	13
5.1 ± 0.1	0.177 ± 0.009	26
7.6 ± 0.2	0.143 ± 0.009	40

As can be seen, potassium iodide affects the rate of ATCH hydrolysis by a considerable amount. Therefore, it is clear that, the percent inhibitions listed in Table XI are not purely due to the action of this inhibitor, but contain a large component due to the iodide interference.

However, the data of Table XII shows that the apparent inhibition due to iodide is linear with the iodide concentration, therefore an estimate of this inhibitor's action can be obtained by adding a value, to the data of Table XI, corresponding to the percent decrease in rate due to iodide at a given concentration of the iodide. The inhibition data corrected in this way is shown in Table XIII.

If this correction is valid; the inhibition data of Table XIII shows that there is no decrease in percent inhibition as the substrate concentration increases and this means non-competitive inhibition. The Km and Vmax values at each inhibitor concentrations were calculated and are listed in Table XIV. This data shows that the Vmax decreases as the inhibitor concentration increases while Km stays constant, implying non-competitive inhibition. It may be pointed out that the Km and Vmax values in the absence of inhibitor are lower and higher, respectively, than the corresponding values listed in Table VI. These values were secured under the same conditions except with different ionic strengths. These values, therefore, show that the lower ionic strengths increase the substrate binding to the enzyme

Table XIII. Data of Table XI corrected for inhibition due to iodide.

ATCH moles/liter) x 10^3	[I] x 10^4 (moles liter^{-1}) 0	2.50 ± 0.07		5.0 ± 0.1		7.5 ± 0.1	
	v	v_i	% inhibition	v_i	% inhibition	v_i	% inhibition
0.212 ± 0.006	0.18 ± 0.01	0.15 ± 0.01	17	0.14 ± 0.01	22	0.13 ± 0.01	28
0.41 ± 0.01	0.27 ± 0.01	0.24 ± 0.01	11	0.21 ± 0.01	22	0.21 ± 0.01	22
0.62 ± 0.01	0.29 ± 0.01	0.27 ± 0.01	7	0.24 ± 0.01	17	0.24 ± 0.01	17
0.83 ± 0.02	0.30 ± 0.02	0.27 ± 0.01	10	0.23 ± 0.01	23	0.23 ± 0.01	23
1.24 ± 0.03	0.33 ± 0.02	0.29 ± 0.01	11	0.26 ± 0.01	21	0.25 ± 0.01*	24

v - velocity in the absence of inhibitor in umoles TCH/min
v_i - velocity in the presence of inhibitor in umoles TCH/min
* - single measurement

Table XIV. Effect of Quaternary nitrogen compound (IV) on the Km and Vmax values.

[I] x 10^4 moles/liter	Km x 10^4 moles/liter	Vmax umoles TCH/min
0	2.2 ± 0.4	0.39 ± 0.02
2.50 ± 0.07	2.4 ± 0.6	0.35 ± 0.02
5.0 ± 0.1	2.2 ± 0.2	0.30 ± 0.02
7.5 ± 0.1	2.3 ± 0.7	0.30 ± 0.03

N.B. Km and Vmax values were calculated from the data of Table XIII.

and, therefore, results in a lower Km value and consequently a higher Vmax.

Most probably the non-competitive inhibition is due to the interaction of the triple bond, a center of high electron density, with the acidic group of the acetylated enzyme, as has been observed with other inhibitors containing a quaternary nitrogen atom and a center of high electron density (25). Some experiments were conducted by incubating the enzyme and this inhibitor for as long as 7 minutes before adding the substrate. No change in percent inhibition, from the ones obtained at zero incubation time, was observed. Therefore any other cause for non-competitive inhibition can be ruled out. However, to be completely

sure that this inhibitor interferes with the deacetylation step (scheme 8), the action of this compound should be studied, using a substrate for which acetylation is the rate determining step.

The data of Table XIII shows that the percent inhibition at 7.5×10^{-4} M and 5×10^{-4} M inhibitor are similar. Ignoring the fact that the iodide interferes and the corrections used may not be correct, the only possible explanation which can be given for this behaviour is as follows: since the inhibitor is an ionic salt, at 7.5×10^{-4} M concentration there may be an appreciable change in the ionic strength and this may be decreasing the percent inhibition.

Because the iodide interferes in the polarographic method, it is possible that the conclusions drawn from the data of Table XIII may not be correct. Therefore, the action of this inhibitor should be studied by some other method or the chloride salt of this compound should be investigated by the polarographic method.

CONCLUSIONS

The inhibition of ACHE by eserine was studied using the polarographic and the spectrophotometric methods. The inhibition was found to be competitive and the two methods gave the similar results. The difference in K_i values and percent inhibition obtained by these methods shows that the ACHE inhibition is strongly dependent on the ionic strength. The fact that the two methods gave similar results shows that the polarographic method can be used for studying ACHE inhibition by eserine and other carbamates.

Though it was reported by Fiserova-Bergerova (17) that the iodide interferes in the polarographic determination of TCH, it has been mentioned in the recent reviews (18) on this subject that the iodide is essential for the polarographic method. In this study, it was found that the iodide interferes due to its anodic wave in the same potential region as the TCH wave. The rates of ATCH hydrolysis, when followed at -0.05 V vs Ag/AgCl, were inhibited considerably by potassium iodide.

Inhibition of ACHE by the quaternary nitrogen compound (IV) was also studied polarographically. After correcting for the inhibition due to iodide, the data showed non-competitive inhibition. This is probably due to the interaction of the triple bond with the acidic group on the acetylated enzyme. Because of iodide interference, the action of this compound should be

re-investigated using the chloride salt or by employing some other method.

SUGGESTIONS FOR FUTURE WORK

The following suggestions for future work can be made:

1. In this study a correction due to the iodide interference was used (see Part III, Results and Discussions). Whether or not this correction can be used should be investigated in detail. This can be done by comparing the rate values obtained with ATCH iodide, after correction for iodide inhibition, and with ATCH chloride. If this correction is valid, the values obtained with the iodide and chloride salts should be the same. The action of the quaternary nitrogen compound (IV) should also be studied by using the chloride salt.

2. There had been some suggestions that acetylcholinesterase may also function as the acetylcholine receptor (3). There are evidences to believe that there is not one but two anionic sites in acetylcholinesterase (3,54). One has a regulatory function (i.e. it responds to the presence of ACH-receptor activators and inhibitors) and the other one is involved in the catalytic process.

The effect of second anionic site on the catalytic functioning of ACHE should be investigated. This can be best done by studying the action of mono- and bis-quaternary nitrogen compounds on the ACHE-catalyzed hydrolysis of the substrate.

Appendix "A"

Buffer solutions used in Polarographic Method.

Phosphate buffers of ionic strength 1.0 or 0.5 and containing 0.01% gelatin as a maximum suppressor were used in all Polarographic measurements and were prepared from 0.2 M stock solutions of $Na_2HPO_4 . 7H_2O$ and $NaH_2PO_4 . H_2O$ [39]

Preparation of pH 7.0 buffer used in Calibration by Glutathione.

To a 76 ml. portion of 0.2 M $Na_2HPO_4 . 7H_2O$ solution, 38 mls. of 0.2 M $NaH_2PO_4 . H_2O$ solution were added to give pH 7.0 measured on E 426 potentiograph. After adjusting pH, 14.9 grams of $NaNO_3$ and 0.025 gram of gelatin were added and the solution was diluted to 250 ml. with boiled deoxygenated distilled water. This yielded a buffer solution of pH 7.0, ionic strength 1.0, and 0.01% gelatin.

Preparation of pH 7.6 buffer used in kinetic experiments.

One liter of buffer solution was normally prepared at a time for use in kinetic experiments as follows: To a 435 ml. portion of 0.2 M $Na_2HPO_4 . 7H_2O$ solution, 65-75 mls. of 0.2 M $NaH_2PO_4 . H_2O$ solution were added to give pH 7.6. Ionic strength was adjusted to 1.0 or 0.5 by adding 59.6 grams or 17 grams of $NaNO_3$. 0.1 gram of gelatin was added to give 0.01% gelatin as maximum suppressor and the solution was diluted to 1 liter with boiled deoxygenated distilled water.

Preparation of Barbital - Phosphate buffer, used in Spectrophoto - metric Method.[40]

A stock solution was prepared by dissolving 2.474 grams of Sodium barbital, 0.272 grams of monopotassium phosphate and 35.07 grams of Sodium Chloride in one liter of distilled water. To a 250 ml. portion of this solution, about 6 mls. of 0.1 N HCl were added to give pH 8.0 and the solution was diluted to 1 liter with distilled water. This solution was used in all spectrophotometric experiments.

Phenol Red Solution.

A 0.01% solution of phenol red prepared in distilled water was used.

Appendix "B"

Calibration using reduced Glutathione (GSH).

15.60 ± 0.02 mg. of reduced GSH (M.W. 307.3) were dissolved in 25 ± 0.03 ml. of boiled distilled water to give a $2.030 \pm 0.004 \times 10^{-3}$ M GSH solution.

Calibration factor was secured by measuring current in ua at - 0.05 V vs Ag/AgCl at two different concentrations of GSH. The solutions composition and current values are listed below.

I. 4.5 ± 0.04 ml. of pH 7.0 buffer + 0.5 ± 0.006 ml. of $2.030 \pm 0.004 \times 10^{-3}$ M GSH giving $2.03 \pm 0.05 \times 10^{-4}$ M GSH.

Run No.	ua	ua/1.00 ± 0.03 x 10^{-4} M GSH
1.	0.284	0.140
2.	0.300	0.148
3.	0.305	0.150
4.	0.300	0.148
5.	0.300	0.148
6.	0.296	0.146

II. 4.00 ± 0.04 ml. of pH 7.0 buffer + 1.00 ± 0.01 ml. of $2.030 \pm 0.004 \times 10^{-3}$ M GSH giving $4.06 \pm 0.09 \times 10^{-4}$ M GSH.

8.	0.620	0.153
9.	0.596	0.147
10.	0.616	0.152

Run No.	ua	ua/1.00 ± 0.03 x 10^{-3} M GSH
11.	0.620	0.153
12.	0.606	0.149

An average value from the above data secured was:

0.149 ± 0.006 ua/$1.00 \pm 0.03 \times 10^{-4}$ M GSH

Calibration by using Acetylthiocholine (ATCH).

5.10 ± 0.02 mg. of ATCH Chloride (M.W.201.7) were dissolved in 25 ± 0.03 ml. of water to give a solution of $1.01 \pm 0.02 \times 10^{-3}$ M ATCH. Calibration factor was secured by measuring current at - 0.05 V vs Ag/AgCl after the complete hydrolysis of ATCH by ACHE. Two different concentrations of ATCH were used, solution composition and current values obtained are listed below.

I. 3.5 ± 0.04 ml. of pH 7.6 buffer + 0.5 ±0.006 ml. of $1.01 \pm 0.02 \times 10^{-3}$ M ATCH + 1.00 ± 0.01 ml. of 1.6 units/ml. ACHE solution. This gave $1.01 \pm 0.04 \times 10^{-4}$ M ATCH in the solution.

Run No.	ua	ua/1.00 ± 0.05 x 10^{-4} M TCH
1.	0.210	0.208
2.	0.215	0.213

II. 3.0 ± 0.04 ml. of pH 7.6 buffer + 1.00 ± 0.01 ml. of $1.01 \pm 0.02 \times 10^{-3}$ M ATCH + 1.00 ± 0.01 ml. of 1.6 units/ml. ACHE solution. This gave $2.02 \pm 0.08 \times 10^{-4}$ M ATCH in the solution.

Run No.	ua	ua/1.00 ± 0.05 x 10^{-4} M TCH
3.	0.392	0.194
4.	0.396	0.196

An average value of the current from the above data was secured as:

$$0.203 \pm 0.009^{*} \text{ ua}/1.00 \pm 0.05 \times 10^{-4} \text{ M TCH}$$

* Standard Deviation.

Appendix "C"

Effect Of Substrate Concentration - Experimental Data

These experiments were carried out in pH 7.6 buffer (Appendix A). The stock solutions of Acetylcholinesterase (ACHE) and Acetylthiocholine (ATCH, M.W. 201.7) were prepared in boiled deoxygenated distilled water. These solutions were kept referigerated and were stable for 4-5 days.

I. [ACHE] = 0.31 ± 0.01 units per 5 ml. of the reaction mixture.

A 0.62 ± 0.02 units/ml. stock solution of ACHE was prepared. In each run 0.5 ± 0.006 ml. of this solution was used, thus giving the concentration of ACHE equal to 0.31 ± 0.01 units per 5 ml. of the reaction mixture.

ATCH stock solutions prepared were as follows:

$1.10 \pm 0.01 \times 10^{-3}$M, $5.85 \pm 0.03 \times 10^{-3}$M, and $1.99 \pm 0.01 \times 10^{-2}$M.

Seven different concentrations of ATCH were studied and the solutions involved are listed below.

(1) 0.25 ± 0.005 ml. of $1.10 \pm 0.01 \times 10^{-3}$M ATCH + 3.75 ± 0.04 ml. of buffer giving $6.1 \pm 0.1 \times 10^{-5}$M ATCH.

(2) 0.5 ± 0.006 ml. of $1.10 \pm 0.01 \times 10^{-3}$M ATCH + 4.0 ± 0.04 ml. of buffer giving $1.10 \pm 0.03 \times 10^{-4}$M ATCH.

(3) 1.00 ± 0.01 ml. of $1.10 \pm 0.01 \times 10^{-3}$M ATCH + 3.5 ± 0.04 ml. of buffer giving $2.20 \pm 0.06 \times 10^{-4}$M ATCH.

(4) 0.5 ± 0.006 ml. of $5.85 \pm 0.03 \times 10^{-3}$M ATCH + 4.0 ± 0.04 ml. of buffer giving $5.8 \pm 0.1 \times 10^{-4}$M ATCH.

(5) 1.00 ± 0.01 ml. of 5.85 ± 0.03 x 10^{-3}M ATCH + 3.5 ± 0.04 ml. of buffer solution giving 1.17 ± 0.02 x 10^{-3}M ATCH.

(6) 0.5 ± 0.006 ml. of 1.99 ± 0.01 x 10^{-2}M ATCH + 4.0 ± 0.04 ml. of buffer giving 1.99 ± 0.05 x 10^{-3}M ATCH.

(7) 1.00 ± 0.01 ml. of 1.99 ± 0.01 x 10^{-2}M ATCH + 3.5 ± 0.04 ml. of buffer giving 3.98 ± 0.09 x 10^{-3}M ATCH.

The initial rates in ua/min., converted to umoles TCH/min, obtained with each of the above seven concentrations of ATCH are listed below:

[ATCH]	ua/min	umoles TCH/min
(1) 6.1 ± 0.1 x 10^{-5}M	0.012	
	0.014	
	0.010	
Average	0.012 ± 0.002	0.030 ± 0.006
(2) 1.10 ± 0.03 x 10^{-4}M	0.018	
	0.018	
	0.018	
Average	0.018 ± 0.000	0.044 ± 0.002
(3) 2.20 ± 0.06 x 10^{-4}M	0.028	
	0.030	
	0.031	
Average	0.030 ± 0.001	0.074 ± 0.005
(4) 5.8 ± 0.1 x 10^{-4}M	0.046	
	0.048	
	0.050	
Average	0.048 ± 0.001	0.118 ± 0.007

[ATCH]	ua/min	umoles TCH/min
(5) $1.17 \pm 0.02 \times 10^{-3}$M	0.056 0.059 0.058 0.048	
Average	0.055 ± 0.003	0.14 ± 0.01
(6) $1.99 \pm 0.05 \times 10^{-3}$M	0.060 0.057 0.055 0.058	
Average	0.056 ± 0.002	0.14 ± 0.01
(7) $3.98 \pm 0.09 \times 10^{-3}$M	0.055 0.055 0.053 0.056	
Average	0.055 ± 0.001	0.14 ± 0.01

N.B. Average deviation and maximum possible error used throughout.

II. [ACHE] = 0.51 ± 0.02 units per 5 ml. of the reaction mixture.

A 1.02 ± 0.03 units/ml. stock solution of ACHE was prepared. In each run 0.5 ± 0.006 ml. of this solution was used, thus giving ACHE concentration of 0.5 ± 0.02 units per 5 ml. of the reaction mixture.

ATCH stock solutions prepared were as follows:

$9.92 \pm 0.05 \times 10^{-4}$M, $4.96 \pm 0.01 \times 10^{-3}$M, and $1.98 \pm 0.01 \times 10^{-2}$M.

Eight different concentrations of ATCH were used. The composition of each solution is listed below.

(1) 0.5 ± 0.006 ml. of $9.92 \pm 0.05 \times 10^{-4}$M ATCH + 4.0 ± 0.04 ml. of buffer giving $9.9 \pm 0.2 \times 10^{-5}$M ATCH.

(2) 1.00 ± 01 ml. of 9.92 ± 0.05 x 10^{-4} M ATCH + 4.0 ± 0.04 ml. of buffer giving 1.98 ± 0.05 x 10^{-4} M ATCH.

(3) 0.4 ± 0.005 ml. of 4.96 ± 0.01 x 10^{-3} M ATCH + 4.1 ± 0.04 ml. of buffer giving 3.97 ± 0.09 x 10^{-4} M ATCH.

(4) 0.6 ± 0.005 ml. of 4.96 ± 0.01 x 10^{-3} M ATCH + 3.9 ± 0.04 ml. of buffer giving 6.0 ± 0.1 x 10^{-4} M ATCH.

(5) 0.8 ± 0.005 ml. of 4.96 ± 0.01 x 10^{-3} M ATCH + 3.7 ± 0.04 ml. of buffer giving 7.9 ± 0.1 x 10^{-4} M ATCH.

(6) 1.00 ± 0.01 ml. of 4.96 ± 0.01 x 10^{-3} M ATCH + 3.5 ± 0.04 ml. of buffer giving 9.9 ± 0.2 x 10^{-4} M ATCH.

(7) 0.5 ± 0.006 ml. of 1.98 ± 0.01 x 10^{-2} M ATCH + 4.0 ± 0.04 ml. of buffer giving 1.98 ± 0.05 x 10^{-3} M ATCH.

(8) 1.00 ± 0.01 ml. of 1.98 ± 0.01 x 10^{-2} M ATCH + 3.5 ± 0.04 ml. of buffer giving 3.96 ± 0.09 x10^{-3} M ATCH.

Initial rates measured as ua/min and converted into umoles TCH/min at each of the above eight concentrations of ATCH are listed below.

[ATCH]	ua/min	umoles TCH/min
(1) 9.9 ± 0.2 x 10^{-5} M	0.028	
	0.034	
	0.030	
Average	0.031 ± 0.002	0.076 ± 0.008
(2) 1.98 ± 0.05 x10^{-4} M	0.056	
	0.053	
	0.052	
	0.052	
Average	0.053 ± 0.001	0.130 ± 0.008

[ATCH]		ua/min	umoles TCH/min
(3) $3.97 \pm 0.09 \times 10^{-4}$ M		0.064	
		0.078	
		0.076	
		0.076	
	Average	0.072 ± 0.005	0.18 ± 0.02
(4) $6.0 \pm 0.1 \times 10^{-4}$ M		0.092	
		0.090	
		0.090	
		0.084	
		0.084	
	Average	0.088 ± 0.003	0.22 ± 0.02
(5) $7.9 \pm 0.1 \times 10^{-4}$ M		0.092	
		0.096	
		0.094	
		0.098	
		0.100	
		0.090	
	Average	0.095 ± 0.004	0.23 ± 0.02
(6) $9.9 \pm 0.2 \times 10^{-4}$ M		0.092	
		0.094	
		0.090	
		0.094	
		0.102	
		0.100	
		0.100	
	Average	0.096 ± 0.004	0.23 ± 0.02
(7) $1.98 \pm 0.05 \times 10^{-3}$ M		0.100	
		0.098	
		0.100	
	Average	0.099 ± 0.001	0.24 ± 0.01
(8) $3.96 \pm 0.09 \times 10^{-3}$ M		0.098	
		0.100	
	Average	0.099 ± 0.001	0.24 ± 0.01

III. [ACHE] = 0.81 ± 0.03 units per 5ml. of the reaction mixture.

A 1.61 ± 0.05 units/ml. stock solution of ACHE was prepared. In each run 0.5 ± 0.006 ml. of this solution was used, thus giving a final ACHE concentration of 0.81 ± 0.03 units per 5 ml. of the reaction mixture.

ATCH stock solutions prepared were as follows:

$1.05 \pm 0.01 \times 10^{-3}$ M, $5.17 \pm 0.03 \times 10^{-3}$ M, and $2.00 \pm 0.01 \times 10^{-2}$ M.

Six different concentration of ATCH were studied and the composition of each solution is listed below.

(1) 0.5 ± 0.006 ml. of $1.05 \pm 0.01 \times 10^{-3}$ M ATCH + 4.0 ± 0.04 ml. of buffer giving $1.05 \pm 0.03 \times 10^{-4}$ M ATCH.

(2) 1.00 ± 0.01 ml. of $1.05 \pm 0.01 \times 10^{-3}$ M ATCH + 3.5 ± 0.04 ml. of buffer giving $2.10 \pm 0.06 \times 10^{-4}$ M ATCH

(3) 0.5 ± 0.006 ml. of $5.17 \pm 0.03 \times 10^{-3}$ M ATCH + 4.0 ± 0.04 ml. of buffer giving $5.2 \pm 0.1 \times 10^{-4}$ M ATCH.

(4) 1.00 ± 0.01 ml. of $5.17 \pm 0.03 \times 10^{-3}$ M ATCH + 3.5 ± 0.04 ml. of buffer giving $1.03 \pm 0.03 \times 10^{-3}$ M ATCH.

(5) 0.5 ± 0.006 ml. of $2.00 \pm 0.01 \times 10^{-2}$ M ATCH + 4.0 ± 0.04 ml. of buffer giving $2.00 \pm 0.05 \times 10^{-3}$ M ATCH.

(6) 1.00 ± 0.01 ml. of $2.00 \pm 0.01 \times 10^{-2}$ M ATCH + 3.5 ± 0.04 ml. of buffer giving $4.0 \pm 0.1 \times 10^{-3}$ M ATCH.

Initial rates in ua/min., converted to umoles TCH/min, obtained with each of the above solution are listed below.

[ATCH]	ua/min	umoles TCH/min
(1) $1.05 \pm 0.03 \times 10^{-4}$ M	0.048	
	0.046	
	0.047	
Average	0.047 ± 0.001	0.12 ± 0.01
(2) $2.10 \pm 0.06 \times 10^{-4}$ M	0.087	
	0.085	
	0.076	
Average	0.083 ± 0.004	0.20 ± 0.02
(3) $5.2 \pm 0.1 \times 10^{-4}$ M.	0.119	
	0.130	
	0.133	
	0.125	
Average	0.127 ± 0.005	0.31 ± 0.02
(4) $1.03 \pm 0.03 \times 10^{-3}$ M	0.169	
	0.165	
	0.168	
	0.168	
Average	0.168 ± 0.001	0.41 ± 0.02
(5) $2.00 \pm 0.05 \times 10^{-3}$ M	0.169	
	0.168	
	0.172	
Average	0.170 ± 0.002	0.42 ± 0.02
(6) $4.0 \pm 0.1 \times 10^{-3}$ M.	0.162	
	0.164	
	0.170	
Average	0.165 ± 0.003	0.41 ± 0.02

N.B. Average Deviation and maximum probable error used throughout.

Appendix "D"

Inhibition by Eserine - Polarographic Method - Experimental Data.

Preparation of Acetylthiocholine Solutions: The four different stock solutions of ATCH prepared were: $1.11 \pm 0.01 \times 10^{-3}$ M, $5.04 \pm 0.01 \times 10^{-3}$ M, $7.50 \pm 0.02 \times 10^{-3}$ M, and $1.988 \pm 0.004 \times 10^{-3}$ M. These were prepared with boiled distilled water.

Preparation of Acetylcholinesterase solution: The ACHE stock solution prepared was 1.03 ± 0.02 units/ml., prepared in boiled distilled water.

Preparation of Eserine Solution: A $4.21 \pm 0.02 \times 10^{-5}$ M stock solution of eserine (M.W. 275.3) was prepared in boiled distilled water.

Twety-four different solutions were studied and the composition of each is given below.

(1) 1.00 ± 0.01 ml. of $1.11 \pm 0.01 \times 10^{-3}$ M ATCH + 3.5 ± 0.04 ml. of buffer giving $2.22 \pm 0.06 \times 10^{-4}$ M ATCH.

(2) 1.00 ± 0.01 ml. of $1.11 \pm 0.01 \times 10^{-3}$ M ATCH + 3.37 ± 0.04 ml. of buffer + 0.130 ± 0.001 ml. of $4.21 \pm 0.02 \times 10^{-5}$ M eserine, giving $2.22 \pm 0.06 \times 10^{-4}$ M ATCH and $1.10 \pm 0.02 \times 10^{-6}$ M eserine.

(3) 1.00 ± 0.01 ml. of $1.11 \pm 0.01 \times 10^{-3}$ M ATCH + 3.25 ± 0.04 ml. of buffer + 0.250 ± 0.001 ml. of $4.21 \pm 0.02 \times 10^{-5}$ M eserine, giving $2.22 \pm 0.06 \times 10^{-4}$ M ATCH and $2.10 \pm 0.04 \times 10^{-6}$ M eserine.

(4) 1.00 ± 0.01 ml. of $1.11 \pm 0.01 \times 10^{-3}$ M ATCH + 3.00 ± 0.04 ml. of buffer + 0.500 ± 0.001 ml. of $4.21 \pm 0.02 \times 10^{-5}$ M eserine, giving

$2.22 \pm 0.06 \times 10^{-4}$ M ATCH and $4.21 \pm 0.07 \times 10^{-6}$ M eserine.

(5) 0.5 ± 0.006 ml. of $5.04 \pm 0.01 \times 10^{-3}$ M ATCH + 4.00 ± 0.04 ml. of buffer giving $5.0 \pm 0.1 \times 10^{-4}$ M ATCH.

(6) 0.5 ± 0.006 ml. of $5.04 \pm 0.01 \times 10^{-3}$ M ATCH + 3.87 ± 0.04 ml. of buffer + 0.130 ± 0.001 ml. of $4.21 \pm 0.02 \times 10^{-5}$ M eserine, giving $5.0 \pm 0.1 \times 10^{-4}$ M ATCH + $1.10 \pm 0.02 \times 10^{-6}$ M eserine.

(7) 0.5 ± 0.006 ml. of $5.04 \pm 0.01 \times 10^{-3}$ M ATCH + 3.75 ± 0.04 ml. of buffer + 0.250 ± 0.001 ml. of $4.21 \pm 0.02 \times 10^{-5}$ M eserine, giving $5.0 \pm 0.1 \times 10^{-4}$ M ATCH and $2.10 \pm 0.04 \times 10^{-6}$ M eserine.

(8) 0.5 ± 0.006 ml. of $5.04 \pm 0.01 \times 10^{-3}$ M ATCH + 3.5 ± 0.04 ml. of buffer + 0.5 ± 0.001 ml. of $4.21 \pm 0.02 \times 10^{-5}$ M eserine, giving $5.0 \pm 0.1 \times 10^{-4}$ M ATCH and $4.21 \pm 0.07 \times 10^{-6}$ M eserine.

(9) 0.5 ± 0.006 ml. of $7.50 \pm 0.02 \times 10^{-3}$ M ATCH + 4.0 ± 0.04 ml. of buffer giving $7.5 \pm 0.2 \times 10^{-4}$ M ATCH.

(10) 0.5 ± 0.006 ml. of $7.50 \pm 0.02 \times 10^{-3}$ M ATCH + 3.87 ± 0.04 ml. of buffer + 0.130 ± 0.001 ml. of $4.21 \pm 0.02 \times 10^{-5}$ M eserine, giving $7.5 \pm 0.2 \times 10^{-4}$ M ATCH and $1.10 \pm 0.02 \times 10^{-6}$ M eserine.

(11) 0.5 ± 0.006 ml. of $7.50 \pm 0.02 \times 10^{-3}$ M ATCH + 3.75 ± 0.04 ml. of buffer + 0.250 ± 0.001 ml. of $4.21 \pm 0.02 \times 10^{-5}$ M eserine, giving $7.5 \pm 0.2 \times 10^{-4}$ M ATCH and $2.10 \pm 0.04 \times 10^{-6}$ M eserine.

(12) 0.5 ± 0.006 ml. of $7.50 \pm 0.02 \times 10^{-3}$ M ATCH + 3.50 ± 0.04 ml. of buffer + 0.5 ± 0.001 ml. of $4.21 \pm 0.02 \times 10^{-5}$ M eserine, giving $7.5 \pm 0.2 \times 10^{-4}$ M ATCH and $4.21 \pm 0.07 \times 10^{-6}$ M eserine.

(13) 1.00 ± 0.01 ml. of $5.04 \pm 0.01 \times 10^{-3}$ M ATCH + 3.50 ± 0.04 ml. of buffer giving $1.01 \pm 0.02 \times 10^{-3}$ M ATCH.

(14) 1.00 ± 0.01 ml. of $5.04 \pm 0.01 \times 10^{-3}$ M ATCH + 3.37 ± 0.04 ml. of

buffer + 0.130 ± 0.001 ml. of $4.21 \pm 0.02 \times 10^{-5}$ M eserine, giving $1.01 \pm 0.02 \times 10^{-3}$ M ATCH and $1.10 \pm 0.02 \times 10^{-6}$ M eserine.

(15) 1.00 ± 0.01 ml. of $5.04 \pm 0.01 \times 10^{-3}$ M ATCH + 3.25 ± 0.04 ml. of buffer + 0.250 ± 0.001 ml. of $4.21 \pm 0.02 \times 10^{-5}$ M eserine giving $1.10 \pm 0.02 \times 10^{-3}$ M ATCH and $2.10 \pm 0.04 \times 10^{-6}$ M eserine.

(16) 1.00 ± 0.01 ml. of $5.04 \pm 0.01 \times 10^{-3}$ M ATCH + 3.00 ± 0.04 ml. of buffer + 0.5 ± 0.001 ml. of $4.21 \pm 0.02 \times 10^{-5}$ M eserine, giving $1.01 \pm 0.02 \times 10^{-3}$ M ATCH and $4.21 \pm 0.07 \times 10^{-6}$ M eserine.

(17) 0.5 ± 0.006 ml. of $1.988 \pm 0.004 \times 10^{-2}$ M ATCH + 4.00 ± 0.04 ml. of buffer giving $1.99 \pm 0.05 \times 10^{-3}$ M ATCH.

(18) 0.5 ± 0.006 ml. of $1.988 \pm 0.004 \times 10^{-2}$ M ATCH + 3.87 ± 0.04 ml. of buffer + 0.130 ± 0.001 ml. of $4.21 \pm 0.02 \times 10^{-5}$ M eserine, giving $1.99 \pm 0.05 \times 10^{-3}$ M ATCH and $1.10 \pm 0.02 \times 10^{-6}$ M eserine.

(19) 0.5 ± 0.006 ml. of $1.988 \pm 0.004 \times 10^{-2}$ M ATCH + 3.75 ± 0.04 ml. of buffer + 0.250 ± 0.001 ml. of $4.21 \pm 0.02 \times 10^{-5}$ M eserine, giving $1.99 \pm 0.05 \times 10^{-3}$ M ATCH and $2.10 \pm 0.04 \times 10^{-6}$ M eserine.

(20) 0.5 ± 0.006 ml. of $1.988 \pm 0.004 \times 10^{-2}$ M ATCH + 3.50 ± 0.04 ml. of buffer + 0.500 ± 0.001 ml. of $4.21 \pm 0.02 \times 10^{-5}$ M eserine, giving $1.99 \pm 0.05 \times 10^{-3}$ M ATCH and $4.21 \pm 0.07 \times 10^{-6}$ M eserine.

(21) 1.00 ± 0.01 ml. of $1.988 \pm 0.004 \times 10^{-2}$ M ATCH + 3.50 ± 0.04 ml. of buffer giving $3.98 \pm 0.09 \times 10^{-3}$ M ATCH.

(22) 1.00 ± 0.01 ml. of $1.988 \pm 0.004 \times 10^{-2}$ M ATCH + 3.37 ± 0.04 ml. of buffer + 0.130 ± 0.001 ml. of $4.21 \pm 0.02 \times 10^{-5}$ M eserine, giving $3.98 \pm 0.09 \times 10^{-3}$ M ATCH and $1.10 \pm 0.02 \times 10^{-6}$ M eserine.

(23) 1.00 ± 0.01 ml. of $1.988 \pm 0.004 \times 10^{-2}$ M ATCH + 3.25 ± 0.04 ml.

of buffer + 0.250 ± 0.001 ml. of $4.21 \pm 0.02 \times 10^{-5}$ M eserine, giving $3.98 \pm 0.09 \times 10^{-3}$ M ATCH and $2.10 \pm 0.04 \times 10^{-6}$ M eserine.

(24) 1.00 ± 0.01 ml. of $1.988 \pm 0.004 \times 10^{-2}$ M ATCH + 3.00 ± 0.04 ml. of buffer + 0.5 ± 0.001 ml. of $4.21 \pm 0.02 \times 10^{-5}$ M eserine, giving $3.98 \pm 0.09 \times 10^{-3}$ M ATCH and $4.21 \pm 0.07 \times 10^{-6}$ M eserine.

N.B. Each solution also contained 0.5 ± 0.006 ml. of 1.03 ± 0.02 units per ml. solution of ACHE. Thus giving 0.51 ± 0.02 units ACHE per 5 ml. of the reaction mixture.

Initial rates obtained with each of the above solution in ua/min and converted to umoles TCH/min are listed below.

Soln. No.	[ATCH]	ua/min	umoles TCH/min
1.	$2.22 \pm 0.06 \times 10^{-4}$ M		
	[I] = 0	0.052	
		0.053	
	Average	0.052 ± 0.001	0.13 ± 0.01
2.	[I] = $1.10 \pm 0.02 \times 10^{-6}$ M	0.041	
		0.044	
		0.041	
	Average	0.042 ± 0.001	0.10 ± 0.01
3.	[I] = $2.10 \pm 0.04 \times 10^{-6}$ M	0.036	
		0.033	
		0.032	
	Average	0.034 ± 0.002	0.08 ± 0.009

* I stands for Esérine.

N.B. Average Deviation and maximum probable error used throughout.

Soln. No.	[ATCH]	ua/min	umoles TCH/min
4.	[I] = 4.21 ± 0.07 x 10^{-6}M	0.026	
		0.022	
	Average	0.024 ± 0.002	0.06 ± 0.01
5.	5.0 ± 0.1 x 10^{-4}M		
	[I] = 0	0.078	
		0.080	
		0.076	
		0.081	
		0.079	
		0.085	
	Average	0.080 ± 0.002	0.20 ± 0.02
6.	[I] = 1.10 ± 0.02 x 10^{-6}M	0.071	
		0.066	
		0.059	
		0.072	
		0.062	
	Avearge	0.066 ± 0.004	0.16 ± 0.02
7.	[I] = 2.10 ± 0.04 x 10^{-6}M	0.058	
		0.060	
		0.054	
		0.058	
		0.062	
	Average	0.058 ± 0.002	0.14 ± 0.01
8.	[I] = 4.21 ± 0.07 x 10^{-6}M	0.044	
		0.049	
		0.050	
		0.049	
		0.046	
	Average	0.048 ± 0.002	0.12 ± 0.01

N.B. Average Deviation and maximum probable error used throughout.

Soln. No.	[ATCH]	ua/min	umoles TCH/min
9.	$7.5 \pm 0.2 \times 10^{-4}$ M		
	[I] = 0	0.100 0.092 0.093 0.100	
	Average	0.096 ± 0.004	0.24 ± 0.02
10.	[I] = $2.10 \pm 0.02 \times 10^{-6}$ M	0.088 0.086 0.084	
	Average	0.086 ± 0.002	0.21 ± 0.02
11.	[I] = $2.10 \pm 0.04 \times 10^{-6}$ M	0.071 0.076 0.071 0.072 0.076	
	Average	0.073 ± 0.002	0.18 ± 0.01
12.	[I] = $4.21 \pm 0.07 \times 10^{-6}$ M	0.065 0.064 0.068	
	Average	0.066 ± 0.002	0.16 ± 0.01
13.	$1.01 \pm 0.02 \times 10^{-3}$ M		
	[I] = 0	0.104 0.104 0.101 0.100 0.098 0.096	
	Average	0.100 ± 0.002	0.25 ± 0.02

Soln. No.	[ATCH]	ua/min	umoles TCH/min
14.	[I] = 1.10 ± 0.02 x 10^{-6}M	0.093	
		0.094	
		0.089	
		0.090	
	Average	0.092 ± 0.002	0.23 ± 0.02
15.	[I] = 2.10 ± 0.04 x 10^{-6}M	0.085	
		0.082	
		0.083	
		0.085	
	Average	0.084 ± 0.001	0.21 ± 0.01
16.	[I] = 4.21 ± 0.07 x 10^{-6}M	0.072	
		0.072	
		0.077	
		0.074	
		0.070	
		0.068	
	Average	0.072 ± 0.002	0.18 ± 0.01
17.	1.99 ± 0.05 x 10^{-3}M		
	[I] = 0	0.105	
		0.103	
		0.105	
		0.100	
	Average	0.103 ± 0.002	0.25 ± 0.02
18.	[I] = 1.10 ± 0.02 x 10^{-6}M	0.094	
		0.095	
		0.095	
		0.093	
		0.092	
	Average	0.094 ± 0.001	0.23 ± 0.01

N.B. Average Deviation and maximum probable error used throughout.

Soln. No.	[ATCH]	ua/min	umoles TCH/min
19.	[I] = 2.10 ± 0.04 x 10^{-6}M	0.087 0.089 0.087 0.087 0.090	
	Average	0.088 ± 0.001	0.22 ± 0.01
20.	[I] = 4.21 ± 0.07 x 10^{-6}M	0.085 0.084 0.086 0.077 0.083 0.078	
	Average	0.082 ± 0.003	0.20 ± 0.02
21.	3.98 ± 0.09 x 10^{-3}M		
	[I] = 0	0.100 0.103 0.100 0.100 0.103	
	Average	0.101 ± 0.001	0.25 ± 0.01
22.	[I] = 1.10 ± 0.02 x 10^{-6}M	0.099 0.101 0.100	
	Average	0.100 ± 0.001	0.25 ± 0.02
23.	[I] = 2.10 ± 0.04 x 10^{-6}M	0.101 0.097 0.099	
	Average	0.099 ± 0.001	0.24 ± 0.01
24.	[I] = 4.21 ± 0.07 x 10^{-6}M	0.092 0.093	
	Average	0.092 ± 0.001	0.23 ± 0.01

Inhibition by Eserine - Spectrophotometric Method - experimental data.

Acetylcholinesterase Solution: A 1.06 ± 0.03 units/ml. stock solution of ACHE was prepared in boiled distilled water.

Acetylthiocholine Solution: The stock solutions of ATCH prepared were: $1.07 \pm 0.01 \times 10^{-3}$ M, $5.06 \pm 0.01 \times 10^{-3}$ M, and $2.003 \pm 0.004 \times 10^{-2}$ M. These were prepared in distilled water.

Eserine Solution: A $4.17 \pm 0.01 \times 10^{-5}$ M stock solution of eserine was prepared in boiled distiiled water.

Twenty different solutions were studied and the composition of each solution is listed below.

(1) 1.00 ± 0.01 ml. of $1.07 \pm 0.01 \times 10^{-3}$ M ATCH + 3.20 ± 0.04 ml. of buffer giving $2.14 \pm 0.06 \times 10^{-4}$ M ATCH.

(2) 1.00 ± 0.01 ml. of $1.07 \pm 0.01 \times 10^{-3}$ M ATCH + 3.07 ± 0.04 ml. of buffer + 0.130 ± 0.001 ml. of $4.17 \pm 0.01 \times 10^{-5}$ M eserine giving $2.14 \pm 0.06 \times 10^{-4}$ M ATCH and $1.08 \pm 0.02 \times 10^{-6}$ M eserine.

(3) 1.00 ± 0.01 ml. of $1.07 \pm 0.01 \times 10^{-3}$ M ATCH + 2.95 ± 0.04 ml. of buffer + 0.250 ± 0.001 ml. of $4.17 \pm 0.01 \times 10^{-5}$ M eserine giving $2.14 \pm 0.06 \times 10^{-4}$ M ATCH and $2.08 \pm 0.03 \times 10^{-6}$ M eserine.

(4) 1.00 ± 0.01 ml. of $1.07 \pm 0.01 \times 10^{-3}$ M ATCH + 2.70 ± 0.04 ml. of buffer + 0.5 ± 0.001 ml. of $4.17 \pm 0.01 \times 10^{-5}$ M eserine giving $2.14 \pm 0.06 \times 10^{-4}$ M ATCH and $4.17 \pm 0.06 \times 10^{-6}$ M eserine.

(5) 0.5 ± 0.006 ml. of $5.06 \pm 0.01 \times 10^{-3}$ M ATCH + 3.70 ± 0.04 ml. of buffer giving $5.1 \pm 0.1 \times 10^{-4}$ M ATCH.

(6) 0.5 ± 0.006 ml. of 5.06 ± 0.01 x 10^{-3} M ATCH + 3.57 ± 0.04 ml. of buffer + 0.130 ± 0.001 ml. of 4.17 ± 0.01 x 10^{-5} M eserine giving 5.1 ± 0.1 x 10^{-4} M ATCH and 1.08 ± 0.02 x 10^{-6} M eserine.

(7) 0.5 ± 0.006 ml. of 5.06 ± 0.01 x 10^{-3} M ATCH + 3.45 ± 0.04 ml. of buffer + 0.250 ± 0.001 ml. of 4.17 ± 0.01 x 10^{-5} M eserine giving 5.1 ± 0.1 x 10^{-4} M ATCH and 2.08 ± 0.03 x 10^{-6} M eserine.

(8) 0.5 ± 0.006 ml. of 5.06 ± 0.01 x 10^{-3} M ATCH + 3.20 ± 0.04 ml. buffer + 0.5 ± 0.001 ml. 4.17 ± 0.01 x 10^{-5} M eserine giving 5.1 ± 0.1 x 10^{-4} M ATCH and 4.17 ± 0.06 x 10^{-6} M eserine.

(9) 1.00 ± 0.01 ml. of 5.06 ± 0.01 x 10^{-3} M ATCH + 3.20 ± 0.04 ml. of buffer giving 1.01 ± 0.02 x 10^{-3} M ATCH.

(10) 1.00 ± 0.01 ml. of 5.06 ± 0.01 x 10^{-3} M ATCH + 3.07 ± 0.04 ml. of buffer + 0.130 ± 0.001 ml. of 4.17 ± 0.01 x 10^{-5} M eserine giving 1.01 ± 0.02 x 10^{-3} M ATCH and 1.08 ± 0.02 x 10^{-6} M eserine.

(11) 1.00 ± 0.01 ml. of 5.06 ± 0.01 x 10^{-3} M ATCH + 2.95 ± 0.04 ml. of buffer + 0.250 ± 0.001 ml. of 4.17 ± 0.01 x 10^{-5} M eserine giving 1.01 ± 0.02 x 10^{-3} M ATCH and 2.08 ± 0.03 x 10^{-6} M eserine.

(12) 1.00 ± 0.01 ml. of 5.06 ± 0.01 x 10^{-3} M ATCH + 2.70 ± 0.04 ml. of buffer + 0.5 ± 0.001 ml. of 4.17 ± 0.01 x 10^{-5} M eserine giving 1.01 ± 0.02 x 10^{-3} M ATCH and 4.17 ± 0.06 x 10^{-6} M eserine.

(13) 0.5 ± 0.006 ml. of 2.003 ± 0.004 x 10^{-2} M ATCH + 3.70 ± 0.04 ml. of buffer giving 2.00 ± 0.05 x 10^{-3} M ATCH.

(14) 0.5 ± 0.006 ml. of 2.003 ± 0.004 x 10^{-2} M ATCH + 3.57 ± 0.04 ml. of buffer + 0.130 ± 0.001 ml. of 4.17 ± 0.01 x 10^{-5} M eserine giving 2.00 ± 0.05 x 10^{-3} M ATCH and 1.08 ± 0.02 x 10^{-6} M eserine.

(15) 0.5 ± 0.006 ml. of 2.003 ± 0.004 x 10^{-2} M ATCH + 3.45 ± 0.04 ml. of buffer + 0.250 ± 0.001 ml. of 4.17 ± 0.01 x 10^{-5} M eserine giving 2.00 ± 0.05 x 10^{-3} M ATCH and 2.08 ± 0.03 x 10^{-6} M eserine.

(16) 0.5 ± 0.006 ml. of 2.003 ± 0.004 x 10^{-2} M ATCH + 3.20 ± 0.04 ml. of buffer + 0.5 ± 0.001 ml. of 4.17 ± 0.01 x 10^{-5} M eserine giving 2.00 ± 0.05 x 10^{-3} M ATCH and 4.17 ± 0.06 x 10^{-6} M eserine.

(17) 1.00 ± 0.01 ml. of 2.003 ± 0.004 x 10^{-2} M ATCH + 3.20 ± 0.04 ml. of buffer giving 4.00 ± 0.09 x 10^{-3} M ATCH.

(18) 1.00 ± 0.01 ml. of 2.003 ± 0.004 x 10^{-2} M ATCH + 3.07 ± 0.04 ml. of buffer + 0.130 ± 0.001 ml. of 4.17 ± 0.01 x 10^{-5} M eserine giving 4.00 ± 0.09 x 10^{-3} M ATCH and 1.08 ± 0.02 x 10^{-6} M eserine.

(19) 1.00 ± 0.01 ml. of 2.003 ± 0.004 x 10^{-2} M ATCH + 2.95 ± 0.04 ml. of buffer + 0.250 ± 0.001 ml. of 4.17 ± 0.01 x 10^{-5} M eserine giving 4.00 ± 0.09 x 10^{-3} M ATCH and 2.08 ± 0.03 x 10^{-6} M eserine.

(20) 1.00 ± 0.01 ml. of 2.003 ± 0.004 x 10^{-2} M ATCH + 2.70 ± 0.04 ml. of buffer + 0.5 ± 0.001 ml. of 4.17 ± 0.01 x 10^{-5} M eserine giving 4.00 ± 0.09 x 10^{-3} M ATCH and 4.17 ± 0.06 x 10^{-6} M eserine.

N.B. Each solution also contained 0.5 ± 0.006 ml. of 1.06 ± 0.03 units/ml ACHE solution and 0.3 ± 0.005 ml. of 0.01% phenol - red solution. Thus giving 0.53 ± 0.03 units of ACHE per 5-ml. of the reaction mixture.

Initial rates in Absorbance units per min. obtained with each of the above solution are listed below.

Soln No.	[ATCH]	Abs. units/min
1.	$2.14 \pm 0.06 \times 10^{-4}$ M	
	[I] = 0	0.037
		0.038
	Average	0.038 ± 0.000
2.	[I] = $1.08 \pm 0.02 \times 10^{-6}$ M	0.027
		0.028
	Average	0.028 ± 0.000
3.	[I] = $2.08 \pm 0.03 \times 10^{-6}$ M	0.021
		0.019
	Average	0.020 ± 0.001
4.	[I] = $4.17 \pm 0.06 \times 10^{-6}$ M	0.014
		0.014
	Average	0.014 ± 0.000
5.	$5.1 \pm 0.1 \times 10^{-4}$ M	
	[I] = 0	0.045
		0.043
		0.044
		0.043
	Average	0.044 ± 0.001
6.	[I] = $1.08 \pm 0.02 \times 10^{-6}$ M	0.035
		0.035
	Average	0.035 ± 0.000

* I stands for Eserine.

Soln No.	[ATCH]	Abs. units/min
7.	$[I] = 2.08 \pm 0.03 \times 10^{-6}$ M	0.029 0.030 0.027
	Average	0.029 ± 0.001
8.	$[I] = 4.17 \pm 0.06 \times 10^{-6}$ M	0.020 0.020
	Average	0.020 ± 0.000
9.	$1.01 \pm 0.02 \times 10^{-3}$ M	
	[I] = 0	0.052 0.050
	Average	0.051 ± 0.001
10.	$[I] = 1.08 \pm 0.02 \times 10^{-6}$ M	0.045 0.046
	Average	0.046 ± 0.000
11.	$[I] = 2.08 \pm 0.03 \times 10^{-6}$ M	0.041 0.038
	Average	0.040 ± 0.002
12.	$[I] = 4.17 \pm 0.06 \times 10^{-6}$ M	0.034 0.034
	Average	0.034 ± 0.000
13.	$2.00 \pm 0.05 \times 10^{-3}$ M	
	[I] = 0	0.049 0.046 0.047
	Average	0.047 ± 0.001

N.B. Average deviation and maximum probable error used throughout.

Soln. No.	[ATCH$_4$]	Abs.units/min
14.	[I] = 1.08 ± 0.02 x 10^{-6} M	0.041 0.039
	Average	0.040 ± 0.001
15.	[I] = 2.08 ± 0.03 x 10^{-6} M	0.038 0.034 0.035
	Average	0.036 ± 0.002
16.	[I] = 4.17 ± 0.06 x 10^{-6} M	0.032 0.034
	Average	0.033 ± 0.001
17.	4.00 ± 0.09 x 10^{-3} M	
	[I] = 0	0.045 0.044
	Average	0.044 ± 0.000
18.	[I] = 1.08 ± 0.02 x 10^{-6} M	0.041 0.041
	Average	0.041 ± 0.000
19.	[I] = 2.08 ± 0.03 x 10^{-6} M	0.038 0.038
	Average	0.038 ± 0.000
20.	[I] = 4.17 ± 0.06 x 10^{-6} M	0.037 0.037
	Average	0.037 ± 0.000

Appendix "E"

Inhibition by 1 - (4 - dimethylamino - 2 - Butynyloxy) - 3 - (2 - Propynyloxy) - Benzene Methiodide using Polarographic Method - Experimental Data.

Acetylthiocholine Solutions. The stock solutions of ATCH Chloride prepared were: $1.06 \pm 0.01 \times 10^{-3}$ M, $4.14 \pm 0.02 \times 10^{-3}$ M and, $6.20 \pm 0.04 \times 10^{-3}$ M. These were prepared in boiled distilled water.

Acetylcholinesterase Solution. A 1.01 ± 0.03 units/ml. stock solution of ACHE was prepared in boiled distilled water. In each run 0.5 ± 0.006 ml. of this solution was used thus giving 0.50 ± 0.02 units of ACHE per 5 ml. of the reaction mixture.

Inhibitor [1 - (4 - dimethylamino - 2 - Butynyloxy) - 3 - (2 - Propynyloxy) - Benzene Methiodide] Solution.

A $5.00 \pm 0.01 \times 10^{-3}$ M stock solution of this inhibitor (M. W. 385.2) was prepared in boiled distilled water.

The inhibition study involved twenty different solutions and the composition of each one is listed below.

(1) 1.00 ± 0.01 ml. of $1.06 \pm 0.01 \times 10^{-3}$ M ATCH + 3.50 ± 0.04 ml. of buffer giving $2.12 \pm 0.06 \times 10^{-4}$ M ATCH.

(2) 1.00 ± 0.01 ml. of $1.06 \pm 0.01 \times 10^{-3}$ M ATCH + 0.250 ± 0.004 ml. of $5.00 \pm 0.01 \times 10^{-3}$ M inhibitor + 3.25 ± 0.04 ml. of buffer giving $2.12 \pm 0.06 \times 10^{-4}$ M ATCH and $2.50 \pm 0.07 \times 10^{-4}$ M inhibitor.

(3) 1.00 ± 0.01 ml. of 1.06 ± 0.01 x 10^{-3} M ATCH + 0.5 ± 0.004 ml. of 5.00 ± 0.01 x 10^{-3} M inhibitor + 3.00 ± 0.04 ml. of buffer giving 2.12 ± 0.06 x 10^{-4} M ATCH and 5.0 ± 0.1 x 10^{-4} M inhibitor.

(4) 1.00 ± 0.01 ml. of 1.06 ± 0.01 x 10^{-3} M ATCH + 0.750 ± 0.004 ml. of 5.00 ± 0.01 x 10^{-3} M inhibitor + 2.75 ± 0.04 ml. of buffer giving 2.12 ± 0.06 x 10^{-4} M ATCH and 7.5 ± 0.1 x 10^{-4} M inhibitor.

(5) 0.5 ± 0.006 ml. of 4.14 ± 0.02 x 10^{-3} M ATCH + 4.00 ± 0.04 ml. of buffer giving 4.1 ± 0.1 x 10^{-4} M ATCH.

(6) 0.5 ± 0.006 ml. of 4.14 ± 0.02 x 10^{-3} M ATCH + 0.25 ± 0.004 ml. of 5.00 ± 0.01 x 10^{-3} M inhibitor + 3.75 ± 0.04 ml. of buffer giving 4.1 ± 0.1 x 10^{-4} M ATCH and 2.50 ± 0.07 x 10^{-4} M inhibitor.

(7) 0.5 ± 0.006 ml. of 4.14 ± 0.02 x 10^{-3} M ATCH + 0.5 ± 0.004 ml. of 5.00 ± 0.01 x 10^{-3} M inhibitor + 3.5 ± 0.04 ml. of buffer giving 4.1 ± 0.1 x 10^{-4} M ATCH and 5.0 ± 0.1 x 10^{-4} M inhibitor.

(8) 0.5 ± 0.006 ml. of 4.14 ± 0.02 x 10^{-3} M ATCH + 0.75 ± 0.004 ml. of 5.00 ± 0.01 x 10^{-3} M inhibitor + 3.25 ± 0.04 ml. of buffer giving 4.1 ± 0.1 x 10^{-4} M ATCH and 7.5 ± 0.1 x 10^{-4} M inhibitor.

(9) 0.5 ± 0.006 ml. of 6.20 ± 0.04 x 10^{-3} M ATCH + 3.5 ± 0.04 ml. of buffer giving 6.2 ± 0.1 x 10^{-4} M ATCH.

(10) 0.5 ± 0.006 ml. of 6.20 ± 0.04 x 10^{-3} M ATCH + 0.25 ± 0.004 ml. of 5.00 ± 0.01 x 10^{-3} M inhibitor + 3.75 ± 0.04 ml. of buffer giving 6.2 ± 0.1 x 10^{-4} M ATCH and 2.50 ± 0.07 x 10^{-4} M inhibitor.

(11) 0.5 ± 0.006 ml. of 6.20 ± 0.04 x 10^{-3} M ATCH + 0.5 ± 0.004 ml. of 5.00 ± 0.01 x 10^{-3} M inhibitor + 3.50 ± 0.04 ml. of buffer giving 6.2 ± 0.1 x 10^{-4} M ATCH and 5.0 ± 0.1 x 10^{-4} M inhibitor.

(12) 0.5 ± 0.006 ml. of 6.20 ± 0.04 x 10^{-3} M ATCH + 0.75 ± 0.004 ml. of 5.00 ± 0.01 x 10^{-3} M inhibitor + 3.25 ± 0.04 ml. of buffer giving 6.2 ± 0.1 x 10^{-4} M ATCH and 7.5 ± 0.1 x 10^{-4} M inhibitor.

(13) 1.00 ± 0.01 ml. of 4.14 ± 0.02 x 10^{-3} M ATCH + 3.5 ± 0.04 ml. of buffer giving 8.3 ± 0.2 x 10^{-4} M ATCH.

(14) 1.00 ± 0.01 ml. of 4.14 ± 0.02 x 10^{-3} M ATCH + 0.250 ± 0.004 ml. of 5.00 ± 0.01 x 10^{-3} M inhibitor + 3.25 ± 0.04 ml. of buffer giving 8.3 ± 0.2 x 10^{-4} M ATCH and 2.50 ± 0.07 x 10^{-4} M inhibitor.

(15) 1.00 ± 0.01 ml. of 4.14 ± 0.02 x 10^{-3} M ATCH + 0.500 ± 0.004 ml. of 5.00 ± 0.01 x 10^{-3} M inhibitor + 3.00 ± 0.04 ml. of buffer giving 8.3 ± 0.2 x 10^{-4} M ATCH and 5.0 ± 0.1 x 10^{-4} M inhibitor.

(16) 1.00 ± 01 ml. of 4.14 ± 0.02 x 10^{-3} M ATCH + 0.750 ± 0.004 ml. of 5.00 ± 0.01 x 10^{-3} M inhibitor + 2.75 ± 0.04 ml. of buffer giving 8.3 ± 0.2 x 10^{-4} M ATCH and 7.5 ± 0.1 x 10^{-4} M inhibitor.

(17) 1.00 ± 0.01 ml. of 6.20 ± 0.04 x 10^{-3} M ATCH + 3.50 ± 0.04 ml. of buffer giving 1.24 ± 0.03 x 10^{-3} M ATCH.

(18) 1.00 ± 0.01 ml. of 6.20 ± 0.04 x 10^{-3} M ATCH + 0.250 ± 0.004 ml. of 5.00 ± 0.01 x 10^{-3} M inhibitor + 3.25 ± 0.04 ml. of buffer giving 1.24 ± 0.03 x 10^{-3} M ATCH and 2.50 ± 0.07 x 10^{-4} M inhibitor.

(19) 1.00 ± 0.01 ml. of 6.20 ± 0.04 x 10^{-3} M ATCH + 0.500 ± 0.004 ml. of 5.00 ± 0.01 x 10^{-3} M inhibitor + 3.00 ± 0.04 ml. of buffer giving 1.24 ± 0.03 x 10^{-3} M ATCH and 5.0 ± 0.1 x 10^{-4} M inhibitor.

(20) 1.00 ± 0.01 ml. of 6.20 ± 0.04 x 10^{-3} M ATCH + 0.750 ± 0.004 ml. of 5.00 ± 0.01 x 10^{-3} M inhibitor + 2.75 ± 0.04 ml. of buffer giving 1.24 ± 0.03 x 10^{-3} M ATCH and 7.5 ± 0.1 x 10^{-4} M inhibitor.

Initial rates obtained in ua/min and converted into umoles TCH/min with each of the solutions are listed below.

Soln. No.	[ATCH]	ua/min	umoles TCH/min
1.	$2.12 \pm 0.06 \times 10^{-4}$ M		
	[I] = 0	0.075 0.076 0.077 0.076 0.068	
	Average	0.074 ± 0.003	0.18 ± 0.01
2.	[I] = $2.50 \pm 0.07 \times 10^{-4}$ M	0.055 0.056 0.048 0.051	
	Average	0.052 ± 0.004	0.13 ± 0.01
3.	[I] = $5.0 \pm 0.1 \times 10^{-4}$ M	0.036 0.038 0.032	
	Average	0.035 ± 0.002	0.086 ± 0.009
4.	[I] = $7.5 \pm 0.1 \times 10^{-4}$ M	0.024 0.023 0.020	
	Average	0.022 ± 0.002	0.054 ± 0.007
5.	$4.1 \pm 0.1 \times 10^{-4}$ M		
	[I] = 0	0.109 0.108 0.106	
	Average	0.108 ± 0.001	0.27 ± 0.01

N.B. Average deviation and maximum probable error are used.

Soln. No.	[ATCH]	ua/min	umoles TCH/min
6.	[I] = 2.50 ± 0.07 x 10^{-4} M	0.086 0.086 0.082	
	Average	0.084 ± 0.002	0.21 ± 0.01
7.	[I] = 5.0 ± 0.1 x 10^{-4} M	0.059 0.061 0.055 0.057	
	Average	0.057 ± 0.002	0.14 ± 0.01
8.	[I] = 7.5 ± 0.1 x 10^{-4} M	0.045 0.042 0.043	
	Average	0.044 ± 0.001	0.11 ± 0.01
9.	6.2 ± 0.1 x 10^{-4} M		
	[I] = 0	0.118 0.117	
	Average	0.118 ± 0.000	0.29 ± 0.01
10.	[I] = 2.50 ± 0.07 x 10^{-4} M	0.094 0.097	
	Average	0.095 ± 0.002	0.23 ± 0.01
11.	[I] = 5.0 ± 0.1 x 10^{-4} M	0.062 0.066	
	Average	0.064 ± 0.002	0.16 ± 0.01
12.	[I] = 7.5 ± 0.1 x 10^{-4} M	0.052 0.050	
	Average	0.051 ± 0.001	0.13 ± 0.01

Soln. No.	[ATCH]	ua/min	umoles TCH/min
13.	$8.3 \pm 0.2 \times 10^{-4}$ M		
	[I] = 0	0.126	
		0.119	
		0.121	
		0.120	
	Average	0.122 ± 0.002	0.30 ± 0.02
14.	[I] = $2.50 \pm 0.07 \times 10^{-4}$ M	0.096	
		0.098	
		0.090	
	Average	0.095 ± 0.003	0.23 ± 0.01
15.	[I] = $5.0 \pm 0.1 \times 10^{-4}$ M	0.060	
		0.062	
		0.056	
		0.057	
		0.059 ± 0.002	0.15 ± 0.01
	[I] = $7.5 \pm 0.1 \times 10^{-4}$ M	0.046	
		0.047	
	Average	0.046 ± 0.000	0.11 ± 0.01
17.	$1.24 \pm 0.03 \times 10^{-3}$ M		
	[I] = 0	0.132	
		0.136	
	Average	0.134 ± 0.002	0.33 ± 0.02
18.	[I] = $2.50 \pm 0.07 \times 10^{-4}$ M	0.104	
		0.100	
		0.101	
	Average	0.102 ± 0.002	0.25 ± 0.01

Soln. No.	[ATCH]	ua/min	umoles TCH/min
19.	[I] = 5.0 ± 0.1 x 10^{-4} M	0.066 0.069	
	Average	0.068 ± 0.002	0.17 ± 0.01
20.	[I] = 7.5 ± 0.01 x 10^{-4} M	0.048	0.12 ± 0.01

N.B. Average deviation and maximum probable error used throughout.

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